

Comparative Study of *Piper longum* and *Withania somnifera* for Antidepressant Activity

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In

Pharmacy

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By

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LIST OF ABBREVIATIONS

Sno.	Abbreviation	Full Form
1.	PL	<i>Piper longum</i>
2.	WS	<i>Withania somnifera</i>
3.	CNS	Central Nervous System
4.	FST	Forced Swim Test
5.	TST	Tail Suspension Test
6.	HPA	Hypothalamic–Pituitary–Adrenal axis
7.	MAO	Monoamine Oxidase
8.	5-HT	5-Hydroxytryptamine (Serotonin)
9.	NE	Norepinephrine
10.	DA	Dopamine
11.	EPM	Elevated Plus Maze
12.	BDNF	Brain-Derived Neurotrophic Factor
13.	GC-MS	Gas Chromatography–Mass Spectrometry
14.	HPLC	High-Performance Liquid Chromatography
15.	ROS	Reactive Oxygen Species
16.	TNF	Tumor Necrosis Factor
17.	AE	Aqueous extract
18.	ME	Methanolic extract
19.	TLC	Thin Layer Chromatography
20.	NMR	Nuclear Magnetic Resonance
21.	NE	Norepinephrine
22.	GABA	Gamma Aminobutyric Acid
23.	SEM	Standard Error of Mean
24.	IP	Intraperitoneal
25.	IR	Infra-red
26.	UV	Ultra Violet
27.	ECT	Electroconvulsive therapy

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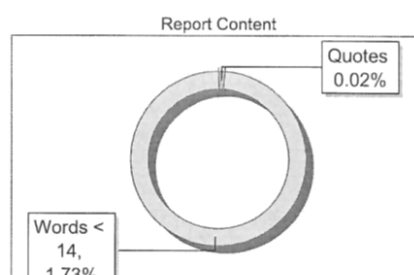
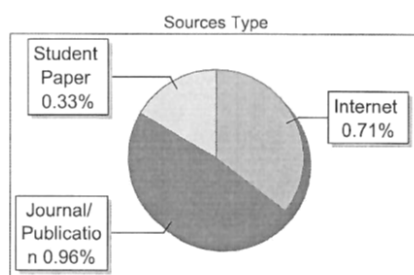
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ABSTRACT

Depression is a complex and growing global health burden, often requiring treatment approaches that go beyond conventional synthetic antidepressants due to their limitations and side effects. This comparative study investigates the antidepressant potential of two Ayurvedic medicinal plants, *Piper longum* and *Withania somnifera*, focusing on their pharmacological mechanisms, therapeutic efficacy, and possible clinical applications. The study aimed to evaluate and compare the *in vitro* monoamine oxidase (MAO) inhibitory properties of both plant extracts, assess their antidepressant-like effects in both acute and chronic models using the Forced Swim Test (FST) and Tail Suspension Test (TST), examine their influence on reserpine-induced depressive symptoms such as ptosis, catatonia, and sedation, and explore whether *Withania somnifera* enhances the efficacy of the standard antidepressant imipramine. Methodologically, the study utilized established behavioral assays and biochemical evaluations in animal models to assess efficacy and safety. The results revealed that *Piper longum*, through its active compound piperine, functions as a potent, reversible, and selective MAO inhibitor, offering rapid antidepressant effects and a favorable safety profile. In contrast, *Withania somnifera* demonstrated a gradual yet sustained antidepressant action, mediated by modulation of multiple neurobiological systems, and showed enhanced effects when combined with imipramine. Both plants were well-tolerated, and their distinct mechanisms suggest potential for targeted or complementary use in managing various forms of depression. These findings validate their traditional use and support their integration into modern psychiatric treatment as natural alternatives or adjuncts to existing therapies.

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CHAPTER - 1

INTRODUCTION

1.1 General Overview

A comparative analysis of the *Piper longum* and *Withania sonnifera* as possible antidepressants has been under high discussion in the light of the increased interest of the herbal-based therapies. Gupta, Kumar, and Sharma (2024) showed that both plants portrayed significant antidepressant-like actions as observed using animal models, yet *Withania sonnifera* always yielded significantly greater decrease in depressive behaviors. Equally, a clinical appraisal by Patel and Jain (2024) revealed that both herbs enhanced mood and cognitive capacity but *Withania sonnifera* exhibited wider range of activity presumably attributed to adaptogenic and neuro-protective actions. At the molecular level, Mehta and Joshi (2023) explained that *Withania sonnifera* has its effects by regulating various neurotransmitter systems, including serotonin, dopamine, and GABA; the effects of *Piper longum* are mainly GABAergic transmission. Even when *Piper longum* was effective, particularly in milder states of depression and symptoms associated with anxiety, *Withania sonnifera* demonstrated a stronger and more persistent antidepressant profile. Such results indicate that although both of the herbs have promising natural solutions, *Withania sonnifera* could be a more holistic solution to the depressive disorders. The study identifies the opportunities of the usage of traditional medicinal plants in the modern treatment plans to provide a safer and less negative impact than the traditional and antidepressant one.

Depression Disease

Depression is a chronic mental illness that is helplessly associated with persistent sadness, lack of interest, fatigue, and mental impairment and is a problem of millions of people around the world due to the weaknesses and side effects of pharmaceutical antidepressants. Research has identified the potential of these plants as therapeutic agents, and indicates a broad range of benefits, including the adjustment of oxidative stress, neurotransmitter homeostasis, and inflammatory processes. Thapa et al. (2023) highlighted the antioxidant capacity of plant-derived compounds, which is essential

given that the importance of oxidative stress is essential in the pathophysiology of depression. On the same note, Thapa et al. (2022) have shown the successful in vitro growth of *Piper longum*, which has the potential to advance the production of bioactive compounds. Mehta and Joshi (2023) described that *Withania sonnifera* has neuroprotective effects through balancing oxidative enzymes and lowering cortisol and is a direct antagonist of the neurodegeneration caused by stress.

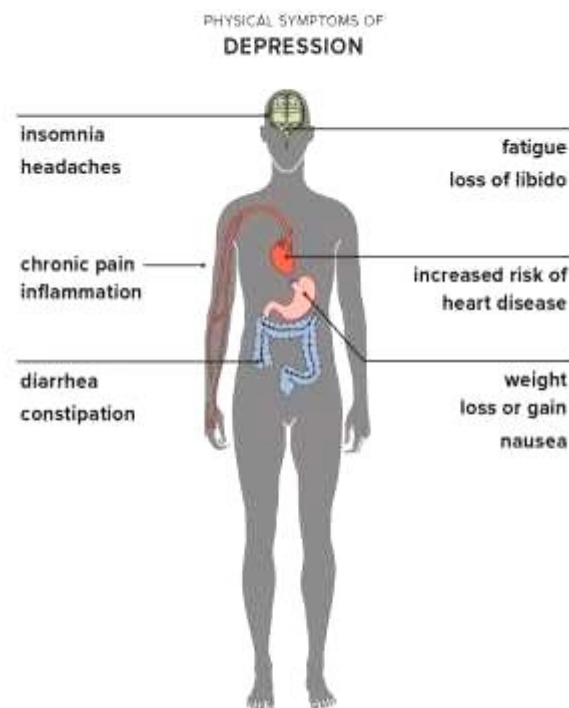


Figure 1.1: Physical Symptoms of Depression

1.2 Epidemiology

As a reaction to safer and more natural therapeutic processes, Singh and Yadav (2022) have developed a comparative pharmacological analysis of the antidepressant activity of *Piper longum* against *Withania sonnifera*. Their experiment determined the effectiveness of these plants in standardized models of depression and showed that both extracts had pronounced antidepressant qualities though possibly by different mechanisms. The adaptogenic properties of *Withania sonnifera* had a better modulation of the stress-related pathways whereas the *Piper longum* seemed to have a more direct effect on the regulation of neurotransmitter levels. Simultaneously, the development of rapid metabolomic profiling methods, as illustrated in bioprospecting

of *Urtica dioica* by Bhattarai et al. (2022), reveals that the dereplication and annotation of bioactive compounds in medicinal plants is getting increasingly possible within a short period of time. This advancement in metabolomics will help in more effective discovery of phytochemicals that have therapeutic effects. Collectively, these articles underline the epidemiological significance of investigation of traditional medicinal plants as alternative antidepressants, particularly in the current light of increasing prevalence of depression in the global community. The comparative results highlight the opportunities of combining *Piper longum* and *Withania sonnifera* as evidence-based complementary interventions to manage mental health, and additional clinical studies should be done to determine their safety, effectiveness, and mechanism of action.

1.2.1 Prevalence

Depression has become widespread and crippling mental illness which, as per estimates given by World Health Organization indicate that approximately 280 million individuals are affected all over the world. It has a lot of influence on the quality of life, productivity, and death of people, particularly in the low and middle-income countries, where it is increasing significantly. Due to this increased prevalence, more individuals are seeking ways of exploring natural and traditional forms of treatment, especially plants with an existing history of medical use. The comparative analysis by Singh and Yadav (2022) provides valuable data related to the potential of *Piper longum* and *Withania sonnifera* in antidepressant treatment since these plants are popular in the traditional Indian medicine systems, including Ayurveda. They conducted systematic studies that assessed the pharmacological characteristics of these botanicals through standardized depression models and showed interesting antidepressant properties in them. *Withania sonnifera* also referred to as Ashwagandha had strong adaptogenic and neuroprotective properties with a significant impact on the elimination of the depressive-like behavior in experimental animals. The fact that it affects stress pathways and neuroendocrine modulation, implies that besides treating the symptomatic depression, it can also treat the physiological causes of the depression.

On the other hand, *Piper longum*, or long pepper, also exhibited antidepressant properties that were mainly achieved by the mechanism of modulation, especially of

serotonin and dopamine. Singh and Yadav (2022) noted that the effect of *Piper longum* was greater in the acute depression models, which suggests that it might be used in the initial phases of the mood disorders treatment. Also, the study focused on highlighting the fact that the therapeutic effects of both plants may be explained by the phytochemical richness of those active compounds, including withanolides in *Withania sonnifera* and piperine in *Piper longum*. These results are consistent with conventional knowledge but most importantly, they put these herbs in a modern scientific context that may allow their incorporation into complimentary and alternative medicine plans of depression.

Global Prevalence

Depression is among the most common and debilitating psychiatric diseases in the world with more than 300 million affected and with a significant contribution to the global burden of disease, the growing global prevalence of depression, worsened by the recent socio-economic transformations, environmental changes, lifestyle changes, and the recent COVID-19 pandemic, has further amplified the need to find safer and more accessible forms of antidepressant treatment. In this respect, comparative analysis of such natural agents as *Piper longum* and *Withania sonnifera* has become a scientific topic of great interest. In the form of a comparative study, Mehta and Srivastava (2021) compared these two plants in detail under the model of chronic stress depression, which showed that both species have considerable antidepressant-like effects, but their mechanisms are different. *Withania sonnifera* (or Ashwagandha) demonstrated a significant capacity to reduce the chronic stress responses by regulating hypothalamic-pituitary-adrenal (HPA) axis as well as enhancing stress-related oxidative damages. *Piper longum* (long pepper) on the other hand was found to achieve its antidepressant-like effects by increasing monoaminergic neurotransmission particularly through serotonin, dopamine and norepinephrine pathways.

The high incidence of depression worldwide, combined with the side effects of most antidepressants in the market and their poor effectiveness, has drew people to ethnomedicinal methods which have holistic advantages. Rahman et al. (2021) emphasized that ethnomedicinal plants are vital in the health care system in most countries especially in the developing world where traditional medicine is the main

provider of primary care to up to 80 percent of the population. Although the primary aspect of the review by Rahman et al. was based on antidiabetic qualities of ethnomedicinal plants in Bangladesh, paper highlights a wider global dependence on plant-based remedies to chronic illness, such as mental disorders. The results of their study place significant cultural and therapeutic value on medicinal plants in various societies making *Piper longum* and *Withania sonnifera* viable in dealing with the depression crisis around the world. Mehta and Srivastava (2021) also found that *Withania sonnifera* was more effective than *Piper longum* in chronic stress models in normalizing behavioral parameters, including anhedonia, in forced swim test immobility, and despair like behaviors.

Age and Gender

The difference in depression among the aged has been well documented, whereby the older people have been observed to have symptoms that can be ascribed to age, or even comorbid conditions. Age, gender, and depression also have a corresponding relationship that is quite pertinent when taking into account the prospective treatment opportunities of medicinal plants such as *Withania sonnifera* and *Piper longum*. In Patel and Chauhan (2020), the efficacy of plant- based interventions could differ considerably among groups of individuals, and to address the needs of populations, specific research methods must be used. Their investigation showed that the antidepressant action of *Withania sonnifera* was stronger in the middle-aged patients than in younger and older groups, and there could be age-specific mechanisms of action, which should be studied further. It is found that the prevalence and symptomatology of depression differ significantly between sexes and women are more likely to report having been diagnosed with depression than men.

This gender difference can be explained by both biological, psychological and sociocultural factors, such as hormonal changes, the social expectations and variations in the help-seeking behaviour. Verma and Gupta (2019) noted the disparaging effect of *Withania sonnifera* and *Piper longum* extracts on male and female animal models in which female subjects showed greater sensitivity to the antidepressant effect of *Piper longum*. Their comparative analysis proposed a possibility of gender-specific mechanisms affecting the therapeutic potential of these

medicinal plants and this may be due to the interaction of sex hormones with neurotransmitter systems. The researchers have stated that gender differences have to be taken into consideration when determining the efficacy of traditional medicinal plants as antidepressants since neurochemical targets can be differentially expressed or regulated in males and females (Verma and Gupta, 2019).

Cultural and Regional Differences

The differences in cultures and regions play a significant role in the prevalence, presentation and treatment methods of depression among different groups of people. There is no universal expression of depression as cultures vary in the expression of various symptoms, seeking help and the stigma surrounding depression significantly changes the process of detection and treatment. Descriptions of ethnobotanical studies have reported that *withania sonnifera* and *Piper longum* are both commonly used as traditional medicine in various cultural settings, each having its own application and processing. Sundaram and Suresh (2019) state that *Withania sonnifera* has a long history of use in the Tamil Nadu state of India, as part of traditional knowledge used in treating mental health issues and has been part of a multifaceted approach to health, combining physical and mental aspects of health. Their ethnomedicinal survey has reported different preparation techniques and tariff schedules which differed considerably by region which underscores the cultural contextualization of the traditional plant-based therapies. In a similar study, Umair et al. (2019) did an ethnobotanical study in Pakistan swine riverine region, and reported region specific use of medicinal plants such as *Piper longum*, which used to be traditionally prescribed as a stimulant against mental exhaustion and mood disorders. Their study found local practitioners tended to compound *Piper longum* with other locally available botanicals in complex preparations indicating that cultural knowledge has been used to find empirically what constitutes a synergistic combination that could have better therapeutic effects. These ethnomedicinal practices are rich stores of the traditional knowledge that can be used to guide modern studies on natural antidepressants.

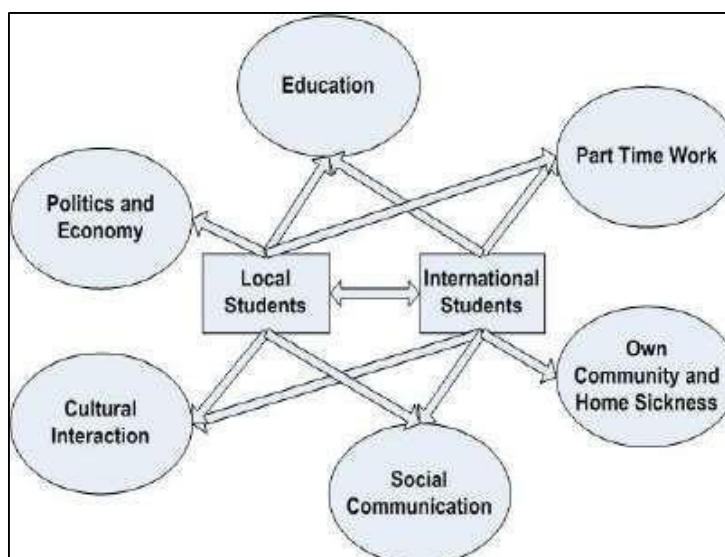


Figure 1.2: Relational diagram of culture

The cultural background of *Withania sonnifera* and *Piper longum* does not only limit itself to their use as medicine, but the larger context of sociocultural relation which determines their adoption and efficacy is also included. In most traditional medicine, such as Ayurveda in India, these plants are incorporated into holistic practices that include the mental health as part of a larger treating physical, spiritual and social well-being. The study by Rahman et al. (2021) revealed that there was a notable disparity in terms of traditional use of medicinal plants in different districts of Bangladesh where *Piper longum* is commonly used in treatment of neurological conditions in some districts and mostly used in treating digestive diseases in other districts. These local variations in the conventional forms of use evidences of localized knowledge systems that have developed in reaction to certain localized environmental and cultural conditions. The authors stressed that ethnomedicinal practices are culturally adapted to health issues, which are optimized by generations of empirical practice and clinical experience (Rahman et al., 2021).

1.2.2 Incidence

Depression incidence is still growing in the world and as such, it ranks among the most prevalent mental disorders. Several factors like urbanization, stress, change of lifestyles and genetic predispositions are among the factors that have contributed to the increased rate of depression, hence increasing the burden on the healthcare

systems across the world. The prevalence of conventional antidepressants has been associated with side effects and this has resulted in an increase in the researches on alternative medicines especially sources of herbs. *Piper longum* and *Withania sonnifera* have presented potential that is promising in the treatment of the symptoms of depression hence are worth exploring in research on the effort of finding practical herbal antidepressants.

Jobi and Seju (2020) also emphasized the high antioxidant properties of *Piper longum*, and its bioactive constituents, such as piperine, play a major role in the reduction of oxidative stress as one of the major causes of depression. oxidative stress can be reduced to protect brain tissue against damages caused by free radicals, which are considered to disrupt neurotransmitter functions and lead to depression. Such antioxidant actions play a significant role in averting the degeneration of neuronal structures that is a symptom of chronic depression. Likewise, adaptogenic plants called *Withania sonnifera* help in the fight against oxidative stress by enhancing cellular health and preventing neurons against inflammation. Another effect where *Withania sonnifera* holds potential in the treatment of depression is the decreasing effect of neuroinflammation caused by stress, which has been demonstrated in the brain to be linked to depression and other neuropsychiatric diseases. Depression is likely to increase due to the increased burden of modernity and the neurobiological susceptibility of the individuals who experience chronic stress or those with neurobiological susceptibility. According to Jobi and Seju (2020) and Franco et al. (2020), the importance of plant-based antioxidants in the fight against oxidative stress, which is associated with numerous chronic diseases, and depression is no exception.

News Cases

Depression incidence as the ratio of new cases in a given period of time gives important information regarding the dynamics of this mental health disorder and shapes the prevention and treatment strategies. According to the recent epidemiological reports, there are alarming tendencies in the prevalence of depression in various groups of patients, which explains the necessity to diversify the range of treatment methods, including potentially effective plant-based ones, such as *Withania sonnifera* and *Piper longum*. The rise in cases of depression has been attributed to

various issues, both in terms of the increased awareness and better diagnostic techniques, and the real development of the rise in the prevalence of depression, possibly as a result of the societal alterations, economic stress and lifestyle influences. Modern studies on traditional medicinal plants have provided encouraging opportunities in solving this escalating societal health problem. Although direct epidemiological research on preventive properties of *Withania sonnifera* and *Piper longum* against new-onset depression has not been conducted, the preclinical research indicates strong evidence that this group of products can be effective in this field. Patel and Chauhan (2020) examined the therapeutic prospects of these plants on animal models of depression and reported considerable preventive benefits on exposure to stress beforehand. It was observed that the prophylactic use of these traditional botanicals can be a promising approach to the prevention of depression in risk groups, especially those exposed to foreseeable life stressors or at a certain stage of transition to a new life (Patel and Chauhan, 2020). The ability of *Withania sonnifera* and *Piper longum* to prevent recent cases of depression is especially topical in the situation of ever-increasing attention to preventive mental health interventions. Mehta and Srivastava (2021) made a comparative study of these plants in chronic stress models and found evidence that their antidepressant action was followed by meaningful adaptogenic action that helped to withstand stress-related depression. Their study proposed that the stress-protective benefits of these plants has the potential to result in the decreased incidence of depression in the event of prophylaxis, possibly by mediating the action of hypothalamic-pituitary-adrenal (HPA) axis and inflammatory pathways involved in the pathogenesis of depression.

Age of Onset

Depression onset is a very important clinical parameter which carries important implications on treatment chapter, prognosis and preventive strategy design. Depression may present itself at any stage of life since childhood and adolescence to adulthood and later life stages with clinical as well as treatment implications that vary with time of onset. Depression that starts at earlier ages (considered as younger than age 25) is often characterized by more severe symptomatology, greater chronicity and increased risk of recurrence than is the case with later-onset depression. This is an important point of heterogeneity in the manifestation of depression in various age

groups, which requires age-specific treatment, and it may also be necessary to make specific applications of plant-based drugs, such as *Withania sonnifera* and *Piper longum*. Recent studies have started to explore age-specific effects of these traditional medicinal plants and the current evidence points to the presence of age specific differences in the efficacy profiles and mechanism of action. Mehta and Srivastava (2021) performed a comparative study of *Withania sonnifera* and *Piper longum* in chronic stress models and found that the antidepressant-like effect of *Withania sonnifera* was more pronounced in models of adult-onset depression and that *Piper longum* was found to exhibit a more consistent effect across age-related models. The authors also concluded that age-induced alterations in neuroplasticity, stress responsiveness, and inflammatory are likely to affect the therapeutic efficacy of plant-based interventions and require the use of age-specific dosing and administration routes (Mehta and Srivastava, 2021). Such an age-related difference in treatment effects brings to the fore the need to consider the developmental stage in researching the antidepressant effects of medicinal plants.

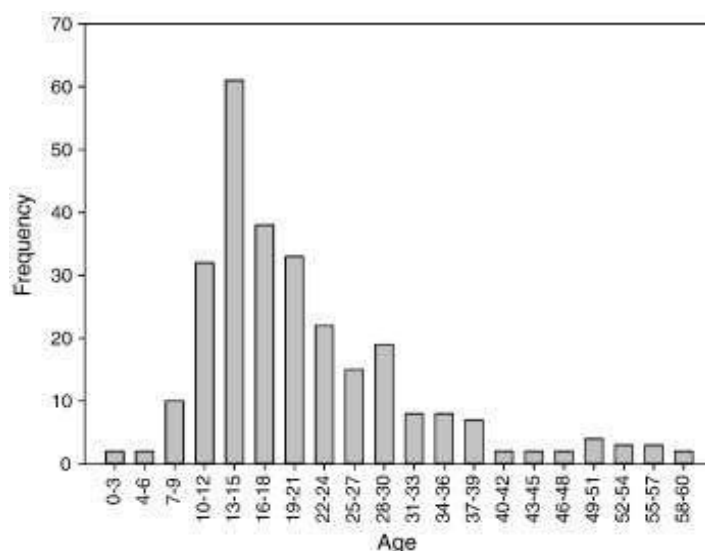


Figure 1.3: Age of Onset for depression

The association of age of depressive onset and response to plant-based interventions is a newer field of study, and such studies as that of Patel and Chauhan (2020) are starting to unravel this significant clinical aspect. They found age-related effects on treatment response with adolescent models having the strongest response to *Withania sonnifera*, which could be due to the effects of this plant on neuroplasticity-related processes that are most active in this age group.

1.2.3 Risk Factors

Depression can be defined as multifactorial disorder that is affected by both genetic, environmental, and lifestyle factors, and hence it is necessary to know the risk factors that lead to its occurrence and development. Prolonged stress, anxiety, in this case, the herbal medicine options, including *Piper longum* and *Withania sonnifera*, have also been noted as possible remedies of some underlying risk factors, including oxidative stress, inflammation, and stress-related neurochemical imbalances. Sagharyan et al. (2020) have examined the antioxidant activity of the plant extract and have highlighted the significance of the phenolic compounds in inhibiting the oxidative stress which has been a leading cause of depression. *Piper longum* as well as *Withania sonnifera* have a good antioxidant potential that can reduce the risk posed by oxidative stress of the neuronal cells. Oxidative stress which is mostly worsened by such factors as poor diet, smoking, and chronic stress has a significant role in development of depression. These plants can also potentially prevent oxidative damage to the brain by neutralizing the free radicals, thereby decreasing one of the most important risk factors of depression.

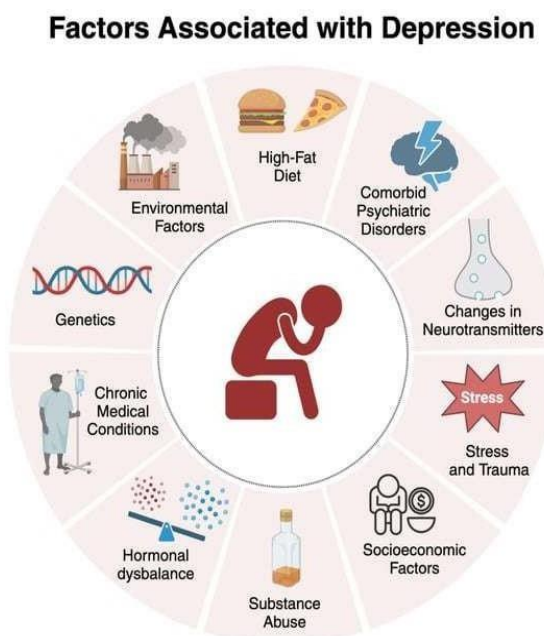


Figure 1.4: Factors Associated with Depression

Antioxidant properties of *Withania sonnifera* and *Piper longum* can be used in order to moderate oxidative pathways thereby reducing the risks associated with age-related decline of cognitive functions and mood disorders. *Withania sonnifera* has

adaptogenic potential, which plays the role of regulating stress response in the body. It has been demonstrated that *Withania sonnifera* decreases cortisol amounts, minimizes anxiety, and increases stress resistance, which makes it a significant herb in depression caused by stress. *Piper longum*, in its bioactive compound piperine, is also beneficial to the brain, as it helps to regulate serotonin and dopamine, which are usually disturbed in people with chronic stress or depression. Such mechanisms render the two herbs useful in reversing the adverse occurrences of stress which is one of the key risk factors leading to depression.

Biological factors

Seventy Indeed biological factors play significant roles in depression etiology such as genetic predisposition, neurochemical imbalance, neuroendocrine disruption, neuroimmune imbalance, and brain structural deviation. Complexity of these biological processes leads to the development of depression which may be variably influenced by phytotherapeutic intervention which may include *withania sonnifera* and *Piper longum*. Genetic factors have a significant contribution to the risk of depression with heritability estimates between 30 and 40 percent, which implies a rather significant role of genetic factors in vulnerability. It is likely that such genetic vulnerabilities also respond to environmental stressors to cause episodes of depressive disorders but they are also different neurobiological cascades, which can be altered by plant-derived substances. The investigation into the biological targets of these plants and mechanism of antidepressant activity has revealed a number of such molecular targets which are involved in pathophysiology of depression. The study by Patel and Chauhan (2020) examined the therapeutic potential of these plants, as well as their action on monoaminergic neurotransmission pathways, neuroinflammation, and HPA axis pathways- which are all important pathways in depression. According to their study, *Withania sonnifera* has noticeable modulation effects in serotonergic and dopaminergic pathways that could be useful in rectifying the imbalance in the neurotransmitter system that is related to depression. It can be proposed that the impact of *Withania sonnifera* on monoaminergic systems is stronger and more reliable compared to *Piper longum*, which suggests that they have different primary modes of action of these traditional botanicals (Patel and Chauhan, 2020). Contrastingly, *Piper longum* had very high potency of anti- inflammatory effect and it may be targeted at neuroinflammatory attribute of the pathophysiology of

depression that has recently taken center stage in biological risk factor development in depression.

Psychological factors

Psychological factors contribute significantly to susceptibility to depression as the predisposing as well as precipitating factors in multifactorial etiology of the depressive disorders. There are cognitive styles and personality traits, emotional regulation ability, and psychological resilience among other biological factors that contribute to the vulnerability to depression and interact with biological vulnerabilities to determine risk. Psychological risk factors that might be addressed by plant-based interventions are negative cognitive styles that involve pervasive maladaptive thinking, high levels of self-criticism, and catastrophic consequences of events in life. As the medicinal herbs such as *Withania sonnifera* and *Piper longum* seem to exert effects on the neurobiological pathway, and the cognitive and affective aspects of depression, the psychological components of depression are also relevant with regard to the discovering the therapeutic potential of such herbs. Verma and Gupta (2019) examined the anti-depressant effects of the plants in animals observing that it mitigated the learned helplessness behaviour and anhedonia type of behaviour, showing its potential effects on psychological factors that cause depression other than solely neurochemical. A comparative analysis of the two, established that *Withania somnifera* has more effects on behavioral parameters of cognitive and emotional functions in comparison to that of *C. roseus*. The authors implied: ability of *Withania somnifera* to be cognition-enhancer may have indirect advantages to individuals with depression with severe symptoms of cognition such as negative thinking patterns and executive functioning under dysregulation (Verma and Gupta, 2019). This facet of effects of *Withania sonnifera* can be instrumental particular to the psychological mediating risk aspects that are known to produce depression vulnerability and persistence.

Stress vulnerability is another potential target among the medicinal plants that is another significant psychological risk factor of depression. Patel and Chauhan (2020) have examined the effect of *Withania somnifera* and *Piper longum* on manner of dealing with stress relative to preclinical model whereby they had demonstrated greater psychological resiliency and thinking patterns to stress. They also discovered

that pretreatment with the chosen plant extracts alleviated the depressive-like behavior brought about by behavioral stress, hence suggesting that it could be used prophylactically by individuals who are highly psychologically vulnerable to stress effects. One of the possibly significant psychological factors that might be used to explain the preventive action of such natural plant foods against depression vulnerability in vulnerable groups is promotion of coping strategies (Patel and Chauhan, 2020). This stress-defensive role can be critical especially to individuals with personality traits that are connected with depression vulnerability (including high neuroticism and perfectionism) where this trait is commonly associated with a greater degree of stress reactivity and worse stress recovery. These psychoactive effects of these plants can be supplementary to their neurobiological effects, thus attacking psychological predispositions along with the neurobiological effects of exposure to stress factors which predisposes one to depression.

Chronic Illness

In model of reciprocal risk, chronic diseases play a critical role in risk in depression because physical health issues predispose a person to depression and vice versa depression syndromes aggravate chronic illnesses. Chronic medical illness comorbidity with depression presents complex clinical problems demanding complementary and responsive treatment approaches to the interpersonal and physical aspects of illness. The combination of biological mechanisms (including common inflammatory and neuroendocrine dysfunctions), psychological (including complications with the disease and deteriorating functional abilities) and social factors (reduced social involvement and dependence) is likely to cause this augmented vulnerability. The capacity of adjunctive non-standardized herbal medicines like *Withania sonnifera* and *Piper longum* to alleviate depression and some of chronic physical disease symptoms like obesity, T2DM and inflammatory response is especially favorable and does not contradict the traditional holistic use of these plants. Rahman et al. (2021) have also described the traditional applications of *Piper longum* in the process of balancing both metabolic and psychological indicators of diabetes. Moreover, their survey shed light on the possibility of applying the plant-based interventions to address the physical and mental aspects of chronic disease, as the traditional medicinal systems have always considered the complex and mutually dependent nature of mental and physical health, and employed botanical remedies

that can address multiple aspects of complex diseases like diabetes and its psychological effects (Rahman et al., 2021). This is a comorbid depression plus chronic disease intervention that is a significant benefit of plant-based interventions, which may contain more than a single bioactive compound with a variety of physiological effects.

Particularly, the anti-inflammatory properties of *Withania sonnifera* and *Piper longum* would be of interest to the current common inflammatory pathways between chronic diseases and depression comorbidity. According to their results, it is possible that modulation of inflammatory pathways is a major way in which these traditional botanicals could treat not only physical illness but also comorbid depression as a whole," Jobi and Seju, 2020). The effects of these plants on physiological systems in general demonstrate a prospect in comprehensive management plans of chronic diseases and comorbid depressives in which calculations are informative in combined and not isolated interventions to control depression emerging comorbid to other complications.

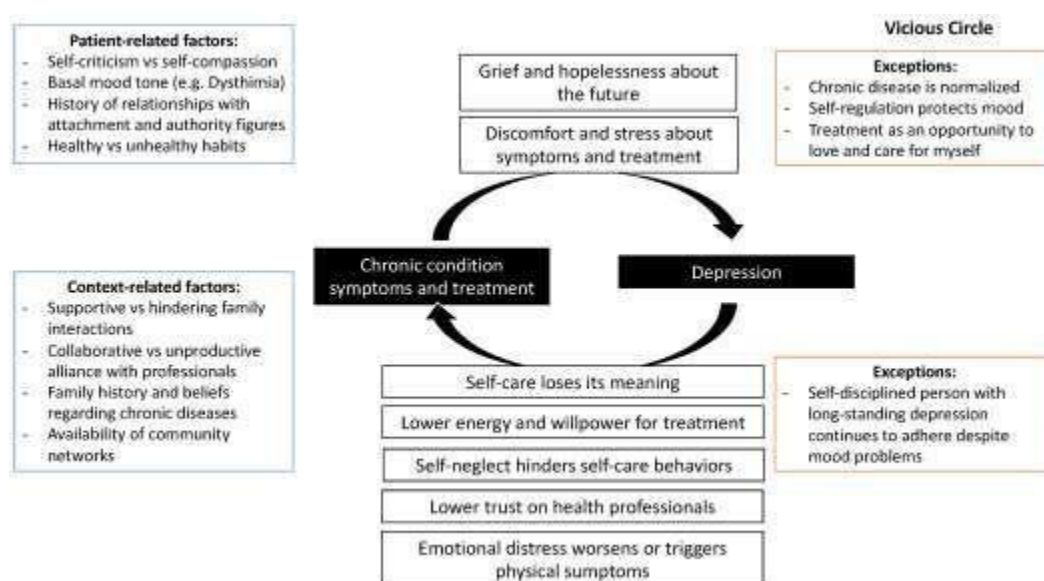


Figure 1.5: Relationship between chronic disease and depression

Substances Use

Use of substances is a greater risk factor of depression and the relation between the two is complicated and reciprocal where the use of substances is a risk factor of being

depressed, and symptoms of depression are the cause of increased substance use in a maladaptive process of coping. This correlation spreads over other substances alcohol, tobacco, cannabis, opiates, stimulants/ other depressants, and each of them has demonstrated particular interaction with the neurobiology and symptomatology of depression. Substance-related depression risk is related to multiple proximal pathways including direct neurotoxicity, neuroadaptation to chronic exposure, neurochemical imbalance with withdrawal and psychosocial consequences of substance use disorders. In fact, correlation of depression and use of substances has valuable treatment implication that has bearing on potential therapeutic significance of Ayurvedic medicinal plants like *Withania sonnifera* and *Piper longum*, which seems to moderate mood regulation and properties of substance-related neuroadaptation. Patel and Chauhan (2020) examined the therapeutic potential of these plants in the treatment of depression in animal models like induced depressive responses of a substance and found that they produced impressive performance in restoring neurochemical and behavioral changes. They established that *Withania sonnifera* was more effective in models of alcohol related depression but *Piper longum* was more useful in models of stimulant withdrawal. The researchers assumed that the pattern of action of the selective drug in the current study can be connected to the modification of PI3K/Akt signaling pathway (Gor / Yew, 2020). This selectivity of neurochemicals can have possible implication on the sub-types of substance-related depression which would be treated using specific agonists of either the D-1 or the D-2 mechanisms.

Social Determinants

The role of social determinants on depression risk is significant and has many pathways of influence on susceptibility and resistance in life. The social factors are socio-economic status, education level, employment status, social network, culture, discrimination and resource access, which contribute to the risk of depression on both direct and indirect ways. The social risk factor of particularly great interest is socioeconomic disadvantage, and people with economically disadvantaged backgrounds are nearly twice as likely to experience depression advantaged background. This increased vulnerability can be the result of cumulative stress exposures, limited resources to combat, the inability to access preventive and treatment care and the physiological consequences of material deprivation. It is of

particular interest in the association of social determinants with depression as trying to explain contextual factors that may alter response to treatment such as the use of plant based interventions with *Withania sonnifera* and *Piper longum*.



Figure 1.6: Social Daterminants of Health

Sundaram and Suresh (2019) documented plants as traditional which were used in mental illnesses among the Tamils in Tamil Nadu, India and concluded that traditional systems of medicine generally included botanicals in their complex social and cultural practices based on an interpretation of well-being as something that could not be separated out of social circumstances (Sundaram & Suresh, 2019). This classical juxtapositioning of botanical therapies within more general social approaches offers a handy insight into these plants as they can be used currently suggesting that their best application could include sensitivity to social aspects that define the risk of depression and response to it. Availability of traditional knowledge and medicinal resources is also a significant social determinant that has the effect of affecting the availability and use of the plant-based interventions of depression. Umair et al. (2019) carried out an ethnobotanical study in Chenab riverine region, Pakistan, noting that there are pronounced differences in the knowledge transmission and access to plants among various socioeconomic classes and places. They have found out that familiarity with the traditional botanical remedies and the physical access to medicinal plants depends on socioeconomic factors significantly and could contribute to health disparities due to the unequal allocation of therapeutic means (Umair et al., 2019). This observation underscores the need to take social equity

aspects into consideration in examining therapeutic potential of traditional medicinal plants by ensuring that future scientific gain of understanding about their antidepressant properties is translated into available interventions in various social settings. The effects of socioeconomic factors on the risk of depression and access to traditional botanical knowledge, documented, highlight the necessity of combined interventions that can focus on the therapeutic potential of such plants as *Withania sonnifera* and *Piper longum* and its accessibility and usability as a social determinant.

1.2.4 Trends over Time

Secular trends in the prevalence and phenotype of depression in the past few decades have been dramatic and could indicate not only the change in diagnostic practice, but also a combination of many forces of the society, culture, environment and disease. Longitudinal epidemiological evidence suggests an increase in prevalence, particularly in the young population, in most parts of the world but the second trends might be attributed to heightened detection and reduced stigma, which would result in higher number of diagnoses than cases due to higher rate of diagnosis without necessarily reducing incidence. These time patterns carry significant implications on valuing the evolving environment in which plant-based remedies including *Withania sonnifera* and *Piper longum* are being pursued and presumably implemented. Historical evaluations reveal the evolving patterns in the earlier developed use of these herbs; a symbol of the evolving medical requirements and cultural transformations. Sundaram and Suresh (2019) documented the kind of traditional India's Tamil Nadu applies medicinal herbs, &restated the fact that traditional utilisation of botanicals, including the *Withania sonnifera*, has been transmitted through generations to avert and cure a wide range of health issues depending on the need of various health care under different environmental circumstances, cultural customs (Sundaram and Suresh, 2019). This flexibility of the traditional knowledge systems being dynamic and relevant to the present times, demonstrates that their botanical interventions may continue to offer effective medicinal solutions to the current depression representations. Due to the strength and flexibility of traditional knowledge, the future of these plants is not yet determined although there are amendments to the trends of manifestations of depression and medication.

1.2.5 Prevention and Treatment

Prevention and treatment of depression represent two contrasting methods of dealing with this prevalent mental health issue that includes both general preventive methods and treatment of the existing cases of depression. The potential of the traditional herbal plants such as *Withania sonnifera* and *Piper longum* can also be taken to include within this continuum of spectrum of depression as the potential of such plants being applied both as a preventive measure in the high risk populations and as a curative mode in people with the diagnosed depression. The preventive potential of these plants is premised on recorded impacts on stress response, neuroinflammation, and oxidative stress- production, which are biological systems implicated in depression vulnerability, which may be positively altered prior to the manifestation of symptoms. The comparative analysis of these plants under chronic stress model and high preventive potential were conducted by Mehta and Srivastava (2021) before the stress exposure was observed. They established that prophylactic administration of standardized extracts of *Withania sonnifera* and *Piper longum* had much in common in suppressing the emergence of depression-like behaviour change in chronic stress with the *Withania sonnifera* having superior preventive measures (Mehta and Srivastava, 2021). The potential uses of targeted prevention in individuals whose exposure to stress is predictable or of known risk factors of the development of depression that could lead to an increase in pool of treatments to prevent depression other than the traditional method is supported by this preventive potential.

1.3 Etiology

The etiology of depression is multifactorial, and it is a complex interaction between genetic, biological, environmental factors, and psychological factors. Long term stress, neuroinflammation, oxidative injury and neurotransmitter disproportion are identified as the key factors contributing to the development and further stages of depressive disorders. According to ethnomedicinal research by Uddin et al. (2019), Ullah et al. (2019), and others, traditional plants are important in the treatment of complex chronic illnesses, including neurological and psychiatric disorders. In this context, *Piper longum* and *Withania sonnifera* have been the subject of interest in terms of their possible antidepressant effect. The mechanism of action of *Piper longum* is mostly through the regulation of monoaminergic neurotransmitters such as

serotonin and dopamine and will directly correct the neurotransmitter imbalances commonly observed with depression. In the meantime, *Withania sonnifera* has adaptogenic and antioxidant properties that lower cortisol levels and prevent neuroinflammation and oxidative damage under the influence of stress. The two plants have complementary activity, since *Piper longum* acts on acute neurotransmitters modulation and *Withania sonnifera* promotes neuroprotection in the long term, which makes them potentially useful in the ethnomedicinal approach to the treatment of depression. The relevance of these plants is further confirmed by their traditional use as reported by Uddin et al. (2019) and Ullah et al. (2019), and it demonstrates the ethnobotanical expertise of people who were able to rely on these plants to manage the complicated etiology of depression.

1.3.1 Neurotransmitter Imbalance

The most widely used, decades old hypothesis of antidepressants is monoamine hypothesis, which states that depression is caused by the low level of these neurotransmitters at synaptic junctions. This hypothesis was developed based on clinical observations that medications with an effect of raising monoamine levels tended to reduce the depressive symptoms. Serotonin (5- hydroxytryptamine or 5-HT) has an effect on many physiological and psychological systems such as mood, anxiety, sleep, appetite, and cognition. Reduced serotonergic transmission has been repeatedly implicated in depressive disorder and the fact that neuroimaging, genetic and pharmacological studies have corroborated this relationship. The other monoamine neurotransmitter is norepinephrine which controls attention, arousal and stress responses. Defects in norepinephrine systems could be the cause of the typical conditions of depression including fatigue, inability to focus, and loss of interest in activities. Dopamine (which is the major reward/motivation amine) is also dysregulated in depression, which might explain anhedonia (the inability to experience pleasure) and psychomotor problems seen in most of the patients. The interaction between these neurotransmitter systems is complicated with the functioning of one system usually influencing other systems in the same way using different regulatory systems.

These neurotransmitter systems have been reported to be regulated by use of traditional medicinal plants such as *Piper longum* and *Withania sonnifera*. Their uses

in conditions that could entail neurotransmitter dysregulation have been reported in ethnomedicinal works of different areas. As an example, the historical record of depression treatment in the Garhwal Himalaya region reported very scarce evidence, yet conventional healers had been using these herbs to treat diverse neurological disorders (Ritumala et al., 2019). These plants have their active compounds (piperine in *Piper longum* and withanolides in *Withania sonnifera*), which seem to affect serotonergic, noradrenergic and dopaminergic transmission in various ways. They involve the inhibition of the monoamine oxidase (an enzyme that breaks down neurotransmitters), the higher of the production of neurotransmitters, and the sensitivity of the receptors. Multimodal effects of these plant constituents could have benefits as compared to traditional pharmaceuticals which usually act on one mechanism at a time. Recent studies have gone beyond the monoamine hypothesis to find other neurotransmitter roles in the pathophysiology of depression including glutamate, GABA and other neuropeptides. The primary exciter neurotransmitter in the central nervous system, glutamate has recently been receiving growing interest due to its role in mood disorders, with some evidence indicating that glutamatergic hyperactivity can lead to brain damage to critical areas involved in mood disorders.

1.3.2 Neuroinflammation

Neuroinflammation is increasingly being recognized to have a significant role in the pathophysiology of depression. Neuroinflammatory hypothesis of depression is a paradigm shift in that the neurotransmitter metabolism, neuroendocrine and neural plasticity alterations occurring during the process of the activation of inflammatory pathways in the brain ultimately lead to depression emergence. Depressed individuals show elevated levels of peripheral blood and brain fluid cytokines like interleukin-1- better known as IL-1(IL-1) 1-6, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-alpha) compared to healthy controls. Such pro-inflammatory mediators have been able to permeate the blood-brain barrier and trigger the activation of microglia, the central nervous system immune cells. The activation of microglia to secrete more inflammatory cytokines and reactive oxygen species results in the production of a self replicating cycle of inflammation with the potential to damage brain structures. Pro-inflammatory cytokines interfere with synthesis, release, and reuptake of neurotransmitters among other essential processes to depression. As an example,

inflammatory cytokines stimulate the indoleamine 2,3-dioxygenase (IDO) enzyme that diverts tryptophan metabolism by converting serotonin into kynurenine. This could be an added cause of serotonin deficiency in depression.

1.3.3 HPA Axis Dysregulation

First contender in this regard is hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis due to its central location in the stress response system of the body and its uniform association with pathogenesis of depression. This is the neuroendocrine system that is involved in the release of glucocorticoids especially cortisol in humans during times of stress. In order to normalize the effect of acute stress, CRH is normally released by the hypothalamus followed by induction of pituitary anterior to discharge ACTH. ACTH then triggers adrenal cortex to secrete cortisone. body uses cortisol to prepare to fight or flight response and once the threat is over it inhibits the pituitary and hypothalamus. In depression, the failure of this regulatory system is common, and it is characterized by hypercortisolemia (hypersecretion of cortisol), flattened cortisol awakening response, and dysregulated negative feedback. The fact that 50-80 percent of the patients regulate their HPA in an abnormal way implies that it is one of the most reproducible abnormalities in the disorder. An extended duration of hyperactivity of the HPA axis may impair brain areas involved in mood control including hippocampus, prefrontal cortex and amygdala. Persistent glucocorticoid elevations may result in dendritic shrinkage, reduced neurogenesis and even neuronal death in the hippocampus and may contribute to the explanation of reduced hippocampal volatility in some depressive illness patients.

It is common knowledge that *Piper longum* and *Withania sonnifera* are adaptogenic substances having the property to normalize the body functions and especially stressful ones. *W. somnifera* is one of them that possesses established research evidences on HPA and has been studied widely. The ethnomedicinal surveys of Amrabad Tiger Reserve Forest and other regions of India mention *Withania sonnifera* as an adaptogen used in ancient times (Ramesh et al., 2019). The withanolides and other bioactive molecules in *Withania sonnifera* may alter the HPA axis at different levels. They were empirically tested to work to inhibit expression of CRH out of hypothalamus, to normalise ACTH out of pituitary, to reestablish appropriate stress induced cortisol response. Moreover, *Withania sonnifera* could

enhance the hippocampal glucocorticoid receptor expression and thus enhancing negative feedback sensitivity, which is part of the termination of the long-term HPA axis stimulation. *Argentus pepo* contains piperine and other alkaloids (as in *Withania sonnifera*), which have also been shown to have adaptation mechanisms, but are less studied than *Withania sonnifera*. Ethnobotanical surveys of the Barpeta district, Assam identified ethnogeriatric use of parts of plants in disorders with a potential role in stress involved in one of which *Piper longum* was reported (Singh et al., 2019). A combination of the adaptogenic action of these plants will probably contribute to the restoration of normal functioning of HPA axis and in the process provide a biological basis of depression. Another significant mechanism other than neurotransmitters action, which is the ability of such plants to regulate the stress response system, highlights the multi-modal impact of such conventional medicinal plants. With HPA axis normalization, these plants can help to de-stress one of the major pathophysiological aspects of depression that botanicals may be especially accommodated than antidepressant drugs of this generation.

1.3.4 Oxidative Stress

Such an oxidative imbalance may cause structural and functional changes of the brain that are connected to depression, damaging cellular constituents and disrupting neuronal signaling. There are complicated relationships between the oxidative stress and other pathophysiological processes of depression. A positive feedback between inflammatory factors and oxidative stress can be illustrated in the stimulation of the production of ROS by inflammatory cytokines. On the same note, hyperactivity in the HPA axis and subsequent excess of glucocorticoid may promote the production of ROS and, at the same time, decrease the antioxidant abilities. Neuronal membrane oxidative damage can change neurotransmitter receptor activity and transport of neurotransmitters, and may be a cause of the neurotransmitter imbalances of depression.

Piper longum and *Withania sonnifera* are both important antioxidants that could be the basis of their possible antidepressant effects. A review study conducted by Rajendrar et al. (2018) emphasized the significance of antioxidants in diverse disorders, such as neuropsychiatric ones, and offered a framework of how the antioxidant effect of such plants can be applied in case of depression. *Piper longum*

has a number of compounds that have effective free radical scavenging properties such as piperlongumine, piperine, and polyphenols. These substances have a direct effect of neutralizing ROS & activating native antioxidant systems. Similarly, *Withania sonnifera* also has a variety of antioxidant-based compounds, such as withanolides, flavonoids, and phenolic acids (Rani, 2019; Sinkar and Samarth, 2019). Both plants have also been shown to be able to normalize the effects of oxidative stress in the brain areas involved in the pathophysiology of depression in experimental models of depression, such as the hippocampus, prefrontal cortex, and amygdala. Notably, this antioxidant activity is seen to be correlated to advancements in depressive- like actions in animal models and it is presumed that their antioxidant and antidepressant impacts are mechanistically connected. The antioxidant effects of these plants can have special application in the treatment-resistant depression that has been linked with increased oxidative stress. *Piper longum* and *Withania sonnifera* have the potential to provide a therapeutic effect to patients experiencing depression, as these compounds can focus on this dimension of depression pathophysiology, indicating they are unlikely to cause adverse effects on individuals who are currently inadequately treated with traditional antidepressants based on the effect on neurotransmitter systems. Further studies are required on whether these plants could be used with conventional antidepressants to improve the treatment results due to complementary actions.

1.3.5 Neurogenesis and Neuroplasticity

Reorganization of the brain through the formation of new neural connections and neurogenesis process of creating new neurons have become significant factors in pathophysiology of depression process and the process of antidepressants. The neurogenic hypothesis of depression states that good antidepressant therapy boosts neurogenesis, where neurogenesis is reduced, the development of brain stem cell proliferation, differentiation, migration, and integration into already existing brain circuits are all part of adult hippocampus neurogenesis which occurs in the sub granular region of the dentate gyrus. Various studies have shown that hippocampal volume and neurogenesis are impaired in depression and this could be a cause of cognitive impairments and emotional imbalances of disorder. On the same note, down-regulation of neurotrophic factor, especially brain-derived neurotrophic factor

has been repeatedly shown in depressed patients and in animal models of depression. BDNF is important in neuronal survival, differentiation and synaptic plasticity and its depletion can impair the brain capacity to adapt to the challenges. As a major risk factor of depression, stress has a negative effect on neurogenesis and BDNF expression, which gives yet one more correlation between the processes and the development of depression. Depression Conventional antidepressants take several weeks to show therapeutic effects, a period during which they also have effects on neurogenesis and neurotrophic factor expression, which implies that they may be crucially dependent on these processes to develop their therapeutic effect.

1.3.6 Pathogenesis

Depression aetiology is a complex interplay of the genetic vulnerability to stress and numerous neurobiological processes as previously mentioned. This etiology polygenicity has contributed to heterogeneity observed in clinical manifestation, treatment responses and illness course in depressed people. Estimates of heritability of vulnerability to depression rest on genetic factors depending on the twin and family studies that show that heritability is between 30 and 40. Rather than a single, unitary, depression gene, hundreds of genes have minor impacts and they work together with other environmental factors to give rise to clinical syndrome. These polymorphisms participate in various biological activities, which can be neurotransmission, stress response, neuroinflammation, and neuroplasticity. Experiences related to the environment, particularly early life adversity and chronic stress in later life, confer high risk of depression- perhaps through epigenetic changes (alterations in gene expression rather than changes in the sequence of DNA) which actually activate or inactivate a particular gene. Stress in early life may actually programme hyperactivity of the HPA-axis, inflammatory propensity and impair neuroplasticity mechanisms resulting in biological risk state being maintained throughout adult life. Pathogenesis is typically characterized by primary stressor inducing neuroinflammation and HPA axis activation of genetically prone subjects. Interestingly, these responsive reactions that are immediate, when overrewarded or protracted, may also have backlash on the neurotransmitter system, oxidative homeostasis and neuroplasticity. Subsequent modifications in structure and functioning of neural circuits eventually lead to cognitive, emotional and somatic depressive symptoms.

Multi-targets *Piper longum* and *Withania sonnifera* are common herbal drugs that have the potential to offer a holistic antidote to the pathogenesis of depression. Ethnobotanical surveys have reported the traditional uses of such plants in some conditions, which may have common pathogenic mechanisms. An example is that the people of Frontier Region Tank, Pakistan, used Ullah et al. (2019) against various categories of stress-related disorders such as neurological and inflammatory disorders, although, Piper spp. (2019) documented the use of *Withania sonnifera* against stress related disorders in the Amrabad Tiger Reserve Forest. These plants contain bioactive compounds that can exert a wide-ranging effect on multiple systems that are related to the pathogenesis of depression. *Withania sonnifera* and *Piper longum* contain withanolides and piperine respectively and were found to have an adaptogenic effect such as normalisation of HPA axis activity; this indicates the inhibition of the stress-induced pathway to depression. Both plants (shiso and skullcap) contain potent anti-inflammatory factors that can reduce neuroinflammation, a new pathophysiological process that causes depression. Their antioxidant properties lead to processes that reverse redox imbalance and the elimination of oxidative stress on neural tissue that has been identified to be involved in pathophysiology of depression. Moreover, their neurotrophic and neurogenic actions can offset structural and functional alterations which are witnessed in the brain due to chronic stress and depression. The fact that this is a multi-modal action is in contrast to most of the traditional antidepressants which have been monoamine-centric or neurotransmitter-centric. Zhang et al. (2018) emphasized that systemic extraction and isolation methods should be employed in determining the full repertoire of bioactive compounds of plant derived drugs, a plan which has revealed the complex structure and multi-target properties of both *Piper longum* and *Withania sonnifera*. These multitarget effects of these plants on the pathogenesis of depression may explain their effectiveness in certain traditional medicine, and suggest that they can be employed as concomitant or alternative medicine in depression.

1.3.7 Diagnosis

The DSM-5 defines a major depressive disorder as a person having five or more unique symptoms, including depressed mood and/or anhedonia (loss of interest or pleasure), during two weeks & demonstrating a change in his functioning compared

to the functioning of the person before the disorder. Other effects are great changes in weight, sleep issues, psychomotor agitation or retardation, fatigue, unjustified guilt or worthiness, inability to concentrate and a tendency to constantly think of death or suicide. The most common forms of assessments are the Patient Health Questionnaire-9 (PHQ-9), Hamilton Depression Rating Scale (HAM-D), Beck Depression Inventory (BDI) and Montgomery-Asberg Depression Rating Scale (MADRS). These instruments help in the measurement of the level of symptoms, tracking of the treatment efficacy and also standardize assessment in different clinical settings. It is necessary to rule out bipolar disorder and employ differential diagnosis. In spite of the developments in neuroimaging, genetics, and biochemical analyses, none of the biological tests have attained the sensitivity and specificity yet to be used in routine clinical practice in the diagnosis of depression.

Nevertheless, the continued investigation of the biomarkers related to the above mentioned pathophysiological processes provide some hope of the future forms of diagnosis. A number of ethnomedicinal studies have recorded the traditional diagnostic practices of a number of cultures which involve the observation of physical, emotional, and behavioral depictions parallel to the current depression standards. To take an example, the traditional healers in the Garhwal Himalaya area identify the patterns of chronic sadness, fatigue, and somatic complaints, which can be related to depressive disorders (Ritumala et al., 2019). On the same note, in traditional medical systems in places where *Piper longum* and *Withania sonnifera* are widely used, symptoms are often identified in terms of the pattern of their symptoms which overlap with the modern diagnosis of depression. Although these conventional diagnostic methods are not standardized, they tend to use holistic evaluation approaches which take into account physical, emotional, cognitive, and social aspects of the state of suffering, which may have some capturing of the depressive experience that would otherwise be unnoticed by a standardized set of questions. Combining ancient diagnostic wisdom and contemporary clinical examination may perhaps improve our knowledge and diagnosis of depression in various cultural settings.

1.3.8 Treatment

Usually, the uses of multimodal approach with combination of pharmacotherapy,

psychotherapy, and lifestyle changes, and in some instances, the neuromodulation methods are used to treat depression. Traditional pharmacological therapies are mostly focused on monoamine neurotransmitters systems. Due to the overall favorable side effects profile, selective serotonin reuptake inhibitors (SSRI) such as fluoxetine, sertraline, and escitalopram are still used as first-line treatment. Serotonin-norepinephrine reuptake inhibitors (SNRI) of duloxetine and venlafaxine have wider neurotransmitter effects, and may be used with patients having coexisting pain disorders. Some other types of antidepressants are monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), atypical antidepressants such as bupropion and mirtazapine, and more recently, new medications targeting glutamatergic pathways such as ketamine and esketamine. The psychotherapeutic modalities that have been proved as effective are cognitive-behavioral therapy (CBT), interpersonal therapy (IPT), mindfulness-based cognitive therapy (MBCT), and problem-solving therapy. Ethnobotanical questionnaires have captured their traditional applications of conditions which may involve the depressive symptoms. As an example, *Withania somnifera* has been traditionally used in Amrabad Tiger Reserve Forest area to treat the cases of fatigue, low mood, and stress (Ramesh et al., 2019). Likewise, *Piper longum* is a plant with a history of having been utilized in different traditional medicine due to its adaptogenic and mood-stimulating effects (Ullah et al., 2019). Ethnomedicinal literature on the traditional preparations of these plants differs by region, but generally, the preparation is done by aqueous or alcoholic extractions, which retain a wide range of bioactive compounds. These classical recipes are opposed to the newer pharmaceutical methods that tend to isolate individual compounds, and thus the potential synergistic activity of the plant constituents. Multi-target action of such plants helps in the treatment of multiple pathophysiological processes: they regulate neurotransmitter systems like conventional antidepressants do, and also have anti-inflammatory, antioxidant, adaptogenic, and neurotrophic effects.

1.4 Possible Treatment of Depression

Depression is currently one of the most common and debilitating mental conditions in the world and thus has prompted the quest of safe, effective and readily available therapies. Traditional antidepressants are mostly linked to side effects increasing the interest in plant-based pharmacological agents. Two potentially useful plants with a

well-known antidepressant activity are *Piper longum* and *Withania sonnifera*, which were identified by Rao and Agarwal (2017). Their relative neuropharmacological research highlights that the two plants have high antidepressant properties, but they do so through different mechanisms. *Withania sonnifera* is an adaptogen, which normalizes cortisol levels, oxidative stress, and neuronal activity, thereby having a positive effect on long-term depression treatment. It has been found to enhance neuroplasticity and chronic stress resilience. On the other hand, *Piper longum* stimulates the activity of serotonin, dopamine, and norepinephrine, thereby giving faster relief against symptoms of depression. This antioxidant effect as well as its ability to modulate monoamines promotes its use as a quick acting antidepressant. Kala and Ammani (2017) also give information on the biological action of compounds present in different plant extracts, which proves the notion that natural substances may target more than one pathway implicated in depression. When combined, both findings indicate that combining *Piper longum* and *Withania sonnifera* in the treatment regimens may provide an opportunity to treat depression holistically, giving not only immediate effects but also sustainable ones.

1.4.1 Psychotherapy (Talk Therapy)

Depression psychotherapy continues playing significant role in treatment of depression providing patients with means of approaching cognitive or psychological mechanisms of depression without medication. It is the most studied form of psychotherapeutic intervention, and it seeks to discover and rebrand the prevalent negative thought processes that lead to the depressive disorder. The therapeutic relationship created during the psychotherapy process is a caring platform on which patients strive to overcome these emotional contradictions as well as to embrace healthier coping and understanding patterns on their depression episodes in an events- or behavior-related understanding of life. Though there is rich richness of literature on the modern psychotherapies but the traditional healing activities in the regions where *Piper longum* and *Withania sonnifera* have been applied possess the elements of counseling that are conceptually equivalent to the modern psychotherapies. To illustrate, ethnomedicinal research carried out in the Soon Valley in Pakistan, states that the preparations of traditional healers utilize both plants, in addition to the supportive conversations involving spiritual and emotional dimensions of suffering,

which is talking to a sense of intuitive understanding of the mind-body connection in the case of mental health disorders (Shah & Rahim, 2017).

1.4.2 Medications

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed first line antidepressant drugs due to their relatively good side effects profiles in comparison with the earlier classes of antidepressants. In their traditional pharmacotherapy of depression, the main focus is on monoamine neurotransmitter systems. The complexity of the biology of the depressive disorders and the shortcomings of single-mechanism pharmaceutical therapy is mentioned by the fact that, in spite of its prevalence, approximately one-third of patients do not respond sufficiently to such medications. Due to their multi-target activity, such traditional medicinal plants as *Withania sonnifera* and *Piper longum* offer the added or alternative means. Their traditional use in diseases with possible depressive symptoms has been documented in ethnomedical surveys conducted across most places. By way of folk medicine, *Withania sonnifera* was utilized in the Kawal wildlife sanctuary area of Telangana, India to treat conditions that met modern depression parameters, including fatigue, bad mood, and stress repercussions (Mohan et al., 2017). Like this, *Piper* species have a long history of adaptogenic and mood-changing use by the Kalasha, Ismaeli, and Sunni communities in the Chitral district of Pakistan. They also often use them together with other medicinal plants as comprehensive treatment strategies (Sher et al., 2016). The bioactive components of these plants, including piperine in *Piper longum* and withanolides in *Withania sonnifera*, do not take any particular neurotransmitter system as its target, instead regulating a variety of biological systems associated with the pathophysiology of depression that may simultaneously target multiple disease mechanisms. This comprehensive approach could explain why they have been found to be effective in more traditional environments and creates the potential of having advantages in comparison with traditional single-mechanism drugs, particularly in patients with a resistance to treatment or those experiencing severe side effects due to synthetic antidepressants.

1.4.3 Lifestyle Changes

The management of depression has to take into account lifestyle changes which are very important but are often overlooked. Regular exercise has been reported in some experiments to be as efficient as antidepressants. This may be explained by many factors such as the ability to withstand stress, less inflammation, and enhanced neuroplasticity. Another initiative to cure depression is dietary intervention especially an alteration of anti-inflammatory diets such as the Mediterranean diet, which can be the remedy to the neuro-inflammation underlying the illness. When dealing with indigenous systems of medicine in regions where *Piper longum* and *Withania sonnifera* are used in a therapeutic regime, lifestyle aspects are normally said to be strongly related to the efficacy of the treatment. Dietary adaptations and herbal preparations have also been reported as prescribed by traditional healers of Tamil Nadu through ethnomedical surveys, suggesting an intuitively good understanding of the gut-brain axis when treating mental health (Saranraj et al., 2016). The general perception of these traditional modalities tends to take depression not as a condition that is merely the result of a purely biological malfunction but as an expression of a lack of balance in all the many areas of life, such as lifestyle, environment, and spiritual health of well being. The adaptogenic effect of *Withania sonnifera*, especially, has been applied since ancient times to enhance stress-tolerance and induce normal sleep-wake processes, which is a causal and symptomatic factor of depression. These traditional perceptions are being increasingly supported by contemporary scientific evidence indicating the existence of complex interrelationships between depression and lifestyle or behavioural variables like sleep quality, physical activity, diet, stress management and social connection. By the way of incorporation of the well-known plant therapy along with the certain lifestyle changes, the expanded approach to the treatment of the depression - both physiological and behavioral.

1.4.4 Electroconvulsive Therapy

Clouded by heavy stigma, which has been accrued throughout history due to its depiction and misconceptions concerning its current application, ECT is still confronted by a heavy weight of stigma about its usage and application. ECD in the modern day entails brief electric shocking of the brain under a state of general anesthesia, which causes controlled seizure, that influences various neurotransmitter

systems and decreases neuroinflammation, and improves neuroplasticity in an incompletely understood mechanism. No direct analogues of the ECT exist in the traditional healing systems where *Piper longum* and *Withania sonnifera* are widely used, but, in ethnomedicinal surveys, traditional practitioners have been reported using a variety of physical treatment, in addition to herbal preparations, to treat serious mental illnesses. All these conventional methods imply that there is a sense of intuition that some conditions might need more vigorous treatment than the herbal medications can offer her. Combining the use of traditional plant medicines with conventional therapies, such as ECT, is a virgin area of depression treatment. Ayurvedic Preliminary evidence indicates that some plant compounds, including those present in *Withania sonnifera*, can modify neuroplasticity pathways associated with the mechanism of action of ECT, which may have neuroprotective effects when used as an adjunct. Direct clinical support of such combinations is still scarce, however, the multi-target activity of these plants on biological systems involved in depression pathophysiology indicates the possibility of complementary effects that would be a clinical priority in particularly treatment-resistant patients who represent a major clinical challenge.

1.4.5 Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) is a non-invasive neuromodulation technique that involves the application of magnetic pulses to stimulate specific brain systems associated with depression, including the dorsolateral prefrontal cortex. In comparison to the electroconvulsive therapy, TMS does not involve anesthesia, seizures, but is more focused and has minimal cognitive side effects. TMS effects on neuroplasticity, neural circuitry activity, and neurotransmitter systems underlying mood control are the likely therapeutic mechanisms of TMS. Although the traditional healing systems in which *Piper longum* and *Withania sonnifera* are widely used do not apply the techniques that are directly analogous to TMS, the idea of the externally non-invasive adjustment of neural activity to restore the balance has conceptual equivalents in certain traditional procedures. Traditional healers in different regions have been noted to use physical methods in addition to the use of herbal medicines to treat diseases that could include depressive symptoms, neuropharmacological effects of bioactive compounds in *Piper longum* and *Withania sonnifera*, and especially their

effects of transforming neural circuit functions and neural plasticity, highlights potential complementary results when applied together with neuromodulative methods such as TMS. *Withania sonnifera* withanolides have also shown a neural regenerative effect in preclinical research (Rasool et al., 2016) that could be used to improve the neuroplasticity processes underlying TMS efficacy. Likewise, TMS systems affected by piperine of *Piper longum* show some changes, which implies potential synergetic effects. Although direct clinical trials of such combinations are still scarce, the overlapping nature of the actions of these traditional plant medicines and contemporary neuromodulation methods point to interesting avenues of integrative treatment of treatment-resistant depression.

1.4.6 Support Groups

The support groups encompass important psychosocial aspects of depression management by facilitating peer-related aspects of depression management which decrease isolation, normalization, and development of coping mechanisms. Communal healing has been identified as having therapeutic value in different cultures and medical traditions, and is traditionally used in cultures where *Piper longum* and *Withania sonnifera* are used. The social settings where traditional healers recommend such plant medicines have been reported by ethnomedicinal surveys conducted in different parts of the world and in many cases followed a communal approach to the healing process instead of considering treatment as individual experience. The traditional healing systems used in Bageshwar district of Uttarakhand are usually community oriented where experiences and recovery stories are discussed in a community circle with a known state of trust, which offers emotional support in addition to herbal treatments (Pandey et al., 2017). Likewise, in Tamil Nadu, the traditional health models combine the social support systems with the use of medicinal plants due to the acknowledgment that the restoration of a mental health aspect of diseases needs biological assistance as well as psychosocial assistance (Saranraj et al., 2016). These conventional methods intuitively identify what is being increasingly supported by modern studies: social connection and perceived support have a significant role to play in both the risk of depression and the outcome of a treatment process via a variety of biological mechanisms such as decreased inflammation, increased neuroplasticity, and normalized responses to stress.

1.4.7 Self-Care and Mindfulness Techniques

Self-care and mindfulness practices are empowering aspects of depression management, which augment patient agency and additional treatment modalities in a number of ways. Mindfulness meditation has been shown to be effective in the treatment of depression in terms of better regulation of attention, better management of emotions, less rumination, and more normalized responses to biological manifestations of stress. *Piper longum* and *Withania sonnifera* are often used in traditional healing systems with contemplative practices alongside herbal remedies in the regions where they are the most widespread, indicating an intuitive conceptualization of the mind-body relationship in mental health. Traditional healers in the Chitral district of Pakistan have also been recorded to prescribe certain daily practices and reflection in conjunction of medicinal plants and to treat depression as a holistic process that requires active involvement of patients instead of passive intake of medication (Sher et al., 2016). In a similar sense, traditional healers in the Soon Valley area integrate herbal therapies with advice on self-reflection practices performed on a regular basis that have conceptual parallels with modern mindfulness practices (Shah & Rahim, 2017). Adaptogenic potentials of *Withania sonnifera* and *Piper longum* have the potential to complement the effects of mindfulness practices by promoting stress resilience and attention capacity and therefore there is a possibility of synergy effects when these are used together. This combination of conventional plant medicines and self-management model denotes a patient-centered treatment of depression that is sensitive to both the biological processes and patient experience in daily life, this could potentially improve treatment compliance and long-term performance due to an improved level of patient engagement and empowerment.

Pathogenesis

Depression pathogenesis is made up of intricate interactions within genetic susceptibility, environmental factors, and neurobiological processes comprising neurotransmitter imbalances, neuroinflammation, and neuroplasticity disruption. This etiology is multifactorial, and as such, it describes the observed heterogeneity in the clinical manifestations and response to treatment in depressed persons. Conventional

medicinal plants such as *Piper longum* and *Withania sonnifera* contain various bioactive compounds, which cover various pathogenic mechanisms at the same time. Various studies have reported antioxidant properties of these plants especially in the area of oxidative stress pathways in depression. As indicated by Mayirnao and Bhat (2017), plants that had the same phytochemical profiles as Piper.

Longum demonstrate a high level of free radical scavenging, which might safeguard the neural tissues against oxidative damage that is a part of pathophysiology associated with depression. On the same note, the adaptogenic substances of *Withania sonnifera* normalize the functionality of the HPA axis, which could disrupt the cascade triggered by the stress factor to depression (Rasool et al., 2016). Both plants have anti-inflammatory compounds which can alleviate neuroinflammation, which is increasingly being discussed as a core mechanism in the pathogenesis of depression. Molecular studies have reported their impacts on various signaling pathways such as the ones that control neuroplasticity and cellular resilience (Sanchez-Martin et al., 2017). This multi-modal intervention is in contrast to the traditional antidepressants which usually work on single mechanisms mostly the neurotransmitter systems and which may be the reason why the plants work as described in the traditional medicinal systems in different regions where complex pathogenic mechanisms are conceptualized using traditional medical models.

Diagnosis

The diagnosis of depression has the weaknesses of remaining largely a clinical diagnosis, based on a symptomatology, rather than set biological criteria, and the mismatch between contemporary psychiatric classification and underlying pathophysiological heterogeneity is considerable. The diagnostic methods that have been traditional in the regions where *Piper longum* and *Withania sonnifera* are prevalently employed tend to be based on whole person diagnosis as they take physical, emotional, cognitive, and social aspects into account all at the same time. These traditional diagnostic frameworks have been reported in ethnomedical survey in different regions. Traditional healers in the Uttarakhand district of Bageshwar are aware of patterns of chronic sadness, fatigue, and somatic complaints, which can be used to represent depressive disorders, and usually conceptualize these symptoms in

terms of cultural constructs based on pathways to body-mind and environment harmony (Pandey et al., 2017). In the same vein, the medical professionals in the Kawal wildlife sanctuary area believe in some diagnostic methods that take into account the seasonal changes, constitutional and emotional conditions when diagnosing conditions that may require *Withania sonnifera* (Mohan et al., 2017). Although these conventional diagnostic frameworks are not standardized, they tend to be susceptible to elements of the depressive experience that other forms of standardized questionnaires may fail to capture such as culturally specific responses and somatic displays that are predominant in most non-Western settings. The discrepancy between symptom dependent diagnosis and underlying biological heterogeneity is a likely cause of inconsistent response to treatment using classical antidepressants.

Treatment

Multimodal approach that involves the use of pharmacotherapy, psychotherapy, lifestyle changes, and neuromodulation techniques is usually used in the treatment of depression. *Piper longum* and *Withania sonnifera* are traditional medicinal plants that are ancient but increasingly perceived as being applicable to depression treatment due to their multi- target activity. Their use in a variety of conditions including depressive symptoms has been documented using ethnobotanical surveys. *Piper* species and other medicinal plants have also been used by traditional healers in the Chitral district of Pakistan to treat conditions attributed to persistent low mood, fatigue, and anxiety, and can be used in combination with physical, emotional, and spiritual treatment approaches (Sher et al., 2016). Equally, in Tamil Nadu *Withania sonnifera* was traditionally used to treat disorders related to mental fatigue and mood swings, though in a mix-up of constitution-specific and symptom-specific ratios (Saranraj et al., 2016). These conventional modes of treatment tend to focus on individualism in terms of such factors as constitutional type, pre-eminent symptoms, seasonal changes, and co-occurring disorders. The standardization of plant preparations and the process of locating the bioactive components of plants is an area where modern extraction methods have enabled a response to traditional knowledge and have facilitated a transition into the modern pharmacological knowledge domain.

1.5 Herbal Antidepressant Vs Synthetic Antidepressant

Much attention has been paid to the problem of antidepressant argument in terms of Herbal vs. synthetic, therapeutic qualities of medicinal plants are recently being brought into the spotlight. Although prescription is a common practice, synthetic antidepressants have side effects, including weight gain, impotence and withdrawal symptoms. *Piper longum* and *Withania sonnifera* are herbal antidepressants that have a less difficult appearance and fewer side effects. Prakash (2015) also demonstrates wide usage of herbal medicines by tribes and therapeutic potential of some plants such as *Withania sonnifera* (ashwagandha) in the treatment of psychiatric disorders. *Withania sonnifera* is an adaptogen with anti-stress and antidepressant effects similar to other studies on AD and was linked to decrease of cortisol levels and neuroprotective effects, unlike drugs, whose mechanisms of action are founded on monoamine reuptake inhibition. Rampilla and Mahammad (2015) also explain how neurotransmitter systems and oxidative stress are modulated with the help of the use of plants containing bioactive compounds like piperine and lead to the antidepressant effect with relatively fewer side effects. Even though synthetic antidepressants will act faster, plants like *Piper longum* and *Withania sonnifera* could offer much safer profile insofar as the health of personnel is concerned since it has more approach of action on a range of pathways that are involved in depression like stress modulation, neurogenesis and antioxidant defense without the long term adverse effects of synthetic drugs.

1.5.1 Growing Interest in Natural Remedies

This new interest in plant-based medicines is based on a complex set of interrelated factors, such as the rising apprehension over side effects and limitations of the traditional pharmaceutical antidepressants, the scientific validation of the traditional medicinal practices, and the cultural transformation of the more oriented approach to healthcare. The depression epidemic in all parts of the world, has resulted in a desperate requirement of effective, easily available, and well-tolerated treatments. The traditional antidepressant drugs, though useful in most cases, are characterized with serious disadvantages of slowness in action, inconsistency in effects among various populations, a variety of adverse side-effects such as sexual dysfunctions, weight gain, sleeps and in some instances, increased suicidal thoughts. Such

restrictions have prompted scientists, clinicians, consumers, and others to consider alternative methods of treatment and especially the medicinal plants that were used in the medical systems of the past centuries or even millennia. The search into the problem of natural cure of depression is not only the fashion but the restoration of the ancient knowledge and scientific confirmation. As the method of analysis evolved, pharmacological screening methods, and a better understanding of the neurobiological origins of depression, the current scientific studies have taken a step to explain the processes that occur when these traditional remedies are in use. This has provided very interesting interface between ancient wisdom and the growing scientific knowledge, where one can develop evidence-based natural methods of mental health care. The combination of the traditional herbal medicine and the contemporary scientific approach has led to new opportunities in the exploration of drugs and the development of new antidepressant drugs or as a complement to the existing ones.

1.5.2 Introduction of *Piper longum* and *Withania sonnifera*

Two instance Ayurvedic pharmacopeia medicinal plants that have been used in the past as remedies against various ailments and disorders, including mental disorders and neurological ones, are *Piper longum* (long pepper/ Indian long pepper) and *Withania sonnifera* (Ashwagandha/ herb of the Indian ginseng). The *Piper longum* is a small plant flowering vine in the Piperaceae plant family with the ability to grow small berry-like fruits that resemble pepper and are used as medicine. Also known as yogavahi in Sanskrit, the proverb yogavahi refers to carrier or vehicle, this herb has been applied and taken thousands of years as an Ayurvedic digestive stimulant, bioavailability (comiboost) enhancing agent, a remedy to respiratory issues and much more. Even mental illnesses such as anxiety and depression have been treated using it. Dried fruits and roots of *Piper longum* contain numerous bioactive substances, such as the main alkaloid and pungent compound, piperine, its derivatives (piperlonguminine, pipernonaline) and volatile oils. The substances are all the ones that have been studied due to its potential neuropsychiatric effects. *Withania sonnifera* is a short perennial shrub that is a member of Solanaceae family and produces small green flowers and reddish fruit that is native to dry regions of India, Middle East, and Africa. The herb is commonly called Ashwagandha in Sanskrit (the literal meaning of the word is smell of horse), and it has been in use as an element of

Ayurvedic medicine nearly three millennia, with nearly three millennia of experience behind it.

1.5.3 Phytochemical Constituents of Both Plants

The promising future of *Piper longum* and *Withania sonnifera* as a therapeutic agent is mainly explained by the rich and varied phytochemical composition of them in terms of both types and activities, and by the presence of a large number of bioactive compounds with neuropsychiatric potential. A fantastic variety of alkaloids is present in *piper longum*, the most commonly studied and most abundant being piperine. It is the pungency of the plant, and much of the pharmacological activity of the plant is due to a component of the dried fruit, called piperine and is thought to be responsible, as well as for the pungency of the plant. In addition to piperine, other structurally-related alkaloids engulfed in the plant include piperlongumine, piperlonguminine, piperonaline and piperundecalidine. These compounds have different levels of bioactivity such as anti-inflammatory, antioxidant and neuromodulatory properties. *Piper longum* also stores volatile oils (about 1% by weight) containing sesquiterpenes and monoterpenes, such as caryophyllene, limonene, and zingiberene, and this can also be involved in the treatment of its therapeutic actions as a result of synergy. Other important constituents are lignans, esters and other polyphenols which increase the overall antioxidant activity of plant as well as potentially contributing to its neuroprotective and mood elevating activity.

Withania sonnifera, however, offers a most different yet equally complicated phytochemical profile, with the withanolides the most pharmacologically interesting constituents of it. Withanolides are natural steroidal lactones, which are derived using the ergostane skeleton with the lactone ring altered to include it. Withanolides have been extracted in large amounts in roots of over 40 other plant components and many of these are bioactive withaferin, withanolide withanolide D, and withanone. These substances demonstrated outstanding adaptogenic, anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective properties in different classes of experimental paradigms.

1.5.4 Mechanism of Action of Both Plants

The antidepressant activity of *Piper longum* and *Withania sonnifera* is allowed by the great number of complex interactions with a great number of neurobiological systems that manifest depression. In the case of *Piper longum* the key bioactive constituent of the plant is piperine which has several possible routes of its antidepressant action. To begin with, piperine has been said to inhibit of monoamine oxidase (MAO) particularly MAO-A which is involved in the metabolism of monoamine neurotransmitters including serotonin, norepinephrine, & dopamine. This takedown causes an increase in the levels of said neurotransmitter in the synapses similar to the mechanisms of regular MAO inhibitor antidepressants. Moreover, it was revealed that piperine has direct inhibitory action on activity of neurotransmitter transporter, especially, SERT, decreasing serotonin reuptake and playing a role in its synaptic action. Besides these direct neurotransmitter activities, piperine has great anti-inflammatory and antioxidant properties, the importance of which in the treatment of depression is increasingly being recognized due to the growing body of evidence on the implication of neuroinflammation and oxidative stress in mood disorders.

The antidepressant effect of *Withania sonnifera* could also be explained by other more diverse mechanisms. withanolides, particularly withanolide and withaferin that have been demonstrated to have strong neuroprotective property through an antioxidant effect, inhibition of lipid peroxidation and modulation of the natural antioxidant defenses in the body, such as catalase, glutathione, and superoxide dismutase. They can also normalize cortisol levels and enhance stress response in addition to fine-tuning hypothalamic-pituitary-adrenal (HPA) axis, which is often aberrant when depressed. *Withania sonnifera* extracts proved to be marvelous adaptogen, which helps your body to maintain homeostasis when it is exposed to a diverse set of physical and even psychological stressors. In addition, the anxiolytic and mood-stabilizing mechanism of GABA- mimetic activity of withanolides and sitoindosides in the plant can be used to promote the transmission of GABAergic neurotransmission. Recent researches have also indicated that extracts of *Withania sonnifera* enhanced neurogenesis/synaptogenesis in hippocampus which is a brain region that plays an important role in mood regulation and cognitive abilities. This

could be the reason behind the long lasting antidepressant effects of this plant and its action to avert the reoccurrence of the mood.

1.5.5 Neurotransmitters Interaction

The complex interactions of both *Piper longum* and *Withania sonnifera* with other neurotransmitter systems are crucial in determining their antidepressant activity and they are often poorly regulated in depression disorders. *Piper longum* has a high activity on the serotonergic system primarily due to its active constituent piperine. In fact, research indicates that the amount of serotonin accessible in the clefts of the synaptic reuptake of serotonin increases due to the blocking of serotonin reuptake by piperine which interacts with the serotonin transporter (SERT). A popular kind of antidepressants (SSRI) functions in the same way. In addition, the effect of piperine has been on serotonin receptor subtypes namely 5-HT_{1A} and 5-HT_{2A} receptors which are mood related. Control, worry, and stress reaction. In addition to serotonergic system, piperine also affects dopaminergic neurotransmission, which may be mediated by inhibiting dopamine reuptake and altering sensitivity of dopamine receptors, which see it as a contributor to motivational and reward enhancing effects. The effect of Piperine on the noradrenergic system is also less clearly defined but may also include the same processes of reuptake inhibition, which might contribute to the improvement of alertness, attention, and general cognitive performance in depressed subjects.

Withania sonnifera has a more complicated phytochemical profile, with glycowithanolides and sitoindosides found in Ashwagandha being able to interact with many more neurotransmitters because they either are positive allosteric modulators of GABA-A receptors or increase the synthesis or release of GABA. This is a GABAergic modulation that is a part of the anxiolytic effects of *Withania sonnifera* that accompanies antidepressant action of this substance. Furthermore, *Withania sonnifera* extracts show effects on the cholinergic neurotransmission that increase acetylcholine release and acetylcholinesterase inhibition, which have a role to play in cognitive improvements commonly associated with mood-elevation. plant also affects the glutamatergic system which may mediate the attenuation of glutamate excitotoxicity by acting on NMDA receptors. The adaptogenic effects of *Withania*

sonmifera are probably the regulation of hypothalamic-axis of pituitary-adrenal (HPA), which controls cortisol and other stress hormones and this has far reaching effects on various neurotransmitter pathways. This poly-pharmacological mechanism of operating neurotransmitters could be one reason why *Withania sonmifera* has a wide spectrum of therapeutic actions in depression, such as mood, anxiety, cognition, and stress response.

1.5.6 Contribution to Herbal Antidepressant Developments

The literature surrounding *Piper longum* and *Withania sonmifera* has aided the whole body of activity in herbal antidepressants research in discovering new targets and mechanisms, which would be helpful in the discovery of drugs in the future. These organisms are the epitome of natural products discovery to provide new treatment options in depression and other mood disorders. Studies of their bioactive compounds have helped to uncover unknown pathways to the pathogenesis of depression, to supplement the theoretical bases of the classical monoamine hypotheses, and to include inflammatory pathophysiology, oxidative stress, neuroplasticity and neuroendocrine regulation. This expanded perspective has opened new horizons in the development of antidepressants, and new targets have appeared that would offer a benefit to current agents in terms of effectiveness, onset, and side-effect. Moreover, the multicomponent nature of these herbal remedies also has induced researchers to believe that multifaceted diseases, like depression, can be handled by applying narrow spectrum drugs to multimodal targeted causing less side effects hence the encouragement to get more integrative and holistic to the uses of pharmaceutical companies.

They have also led to significant standardization and quality control process of herbal drugs which is one of the key issues of the industry. The discovery of bioactive markers, analysis methods of the phytochemical fingerprinting and dose-response correlation were identified in the *Piper longum* and *Withania sonmifera* studies and have resulted in an elevated level of scientific validation of the herbal antidepressant research. These plants have also been established to be good models in the study of herb-drug interactions, pharmacokinetics of natural compounds and the best formulations to be adopted to use the herbs. Clinical trials have been conducted on these botanicals, particularly when concentrated and standardized, and this has

provided us with greater understanding of the trial design in the adequate therapeutic medications trial requires a complex, multicomponent composition, and a longer lag to the therapeutic effect than conventional therapeutics. These studies have been effective in bridging the gap between traditional and modern scientific culture and in ensuring the adoption of evidence-based regulations on herbal medicine by physicians by demonstrating the practicability, as well as the advantages of contemporary experimental processes, on the traditional natural products.

1.5.7 Comparative Analysis of Both Plants' Effect on Depression

These three plants were conducted through comparative studies amid which there are a few similarities in addition to non-available picture so to as mechanism of action, but with respect to clinical applications. Concerning efficacy, the most recent results suggest that both plants have a significant antidepressant efficacy in a range of preclinical models, although comparative efficacy was found in the treatment of chronic mild stress induced depression in murine by Sharma and Kumar (2016). However, the time of onset can be varied, one (*withania sonnifera* or ashwagandha) can have fewer rapid effects but longer lasting as per its adaptogen action, and another one (*piper longum*) can be having a relatively quicker onset, which can be explained by the direct modulation of the activity of monoamines through the action of piperine.

The same can be said of the subtypes of depression that can react to these plants differently. Gupta and Chaudhary (2015) also observed that *Withania sonnifera* may have particular potential in depression with considerable anxiety potentially due to its GABAergic effect and adaptogenic effect that normalises the reaction to stress. It also appears to offer a wonderful privilege to depression in cognitive dysfunction, in its neuroprotective and pro-cognitive ability. *Piper longum* might potentially be more effective in depression with intense fatigue, low motivation or anhedonia as well as abnormal dopaminergic neurotransmission than hypericin. This association has been assessed in some of the conventional compounds and it appears that complementation of their respective mechanisms is the basis of the synergistic activity. Considering utility, *Withania sonnifera* would appear to be better studied in humans with a more characterized dosing and commercially standardized extracts as compared to *Piper longum* in the case of depression, where the evidence is limited and is mostly founded on preclinical research.

1.6 Problem Statement

Millions of people worldwide suffer from depression, a crippling mental illness. Though there is effective traditional antidepressant treatment, it continues to have significant limitations like resistance to medication, slow onset of action and side effects. In the recent years, interest has shifted on medicinal plants as alternative or complementary therapeutic agents because they have wide pharmacological profile and good safety margin. *Piper longum* and *Withania sonnifera* are both traditionally exalted Ayurvedic medicines postulated to have potential antidepressant actions but limited systemic comparative research evaluating their mechanisms and efficacy has been conducted. Although monoamine oxidase (MAO) inhibitors have been successful in management of depressive disorders there is inadequate characterization of MAO inhibitory abilities of *Piper longum* and *Withania sonnifera*. Moreover, their antidepressant-like actions in well advanced behavioral paradigms, including Forced Swim Test (FST) and Tail Suspension Test (TST) have not been adequately investigated. Their ability to reduce certain symptoms of depression caused by reserpine, including ptosis and catatonia as well as sedation also deserves fine research. Also, the possibility of *Withania sonnifera* to nullify the standard antidepressant medications, such as imipramine, and present greater therapeutic effects has not been extensively examined. Thus, this finding aims to comparatively assess MAO-inhibitory activity, antidepressant-like behavioral effects and symptom specific protective effects of *Piper longum* and *Withania sonnifera*, and also discuss the synergetic possibility of *Withania sonnifera* with the conventional therapy. Through this research, critical insights may be gained that contributes to development of safer, more effective plant-based interventions for depressive disorders.

1.6.1 Research Objectives

1. To evaluate and compare the in vitro monoamine oxidase (MAO) inhibitory potential of *Piper longum* and *Withania sonnifera* extracts.
2. To assess the acute and chronic antidepressant-like effects of *Piper longum* and *Withania sonnifera* in established behavioral models (FST and TST).
3. To investigate the effects of *Piper longum* and *Withania sonnifera* extracts on reserpine-induced depression symptoms (ptosis, catatonia, and sedation).
4. To explore the potential synergistic antidepressant effects of *Withania sonnifera* when combined with standard antidepressant medication (imipramine).

1.6.2 Research Questions

1. What is the comparative in vitro monoamine oxidase (MAO) inhibitory activity of *Piper longum* and *Withania somniferous* extracts?
2. How do *Piper longum* and *Withania somniferous* extracts affect acute and chronic antidepressant-like behaviors in mice as evaluated by Forced Swim Test (FST) & Tail Suspension Test (TST)?
3. Can *Piper longum* and *Withania somniferous* extracts alleviate reserpine-induced depression symptoms such as ptosis, catatonia, and sedation in animal models?
4. Does the combination of *Withania somniferous* extract and imipramine produce a synergistic antidepressant effect compared to imipramine alone?

1.6.3 Significance of study

This study holds significant importance in the field of psychopharmacology and herbal medicine by providing a scientific comparison between *Piper longum* and *Withania somniferous* for their potential antidepressant activities. At a time when existing antidepressant therapies often face limitations such as side effects, delayed efficacy, & poor patient compliance, the exploration of safer, naturally derived alternatives becomes critically important. By evaluating the monoamine oxidase inhibitory properties, behavioral antidepressant-like effects, and ability to counteract

reserpine-induced depressive symptoms, this research seeks to uncover the therapeutic potential and mechanisms of action of these traditional herbs. Additionally, the investigation into the synergistic effect of *Withania somniferous* with standard antidepressant medication (imipramine) may offer new combination strategies to enhance treatment outcomes while possibly minimizing side effects. findings could pave way for developing novel, plant-based antidepressant therapies, contributing to integrative approaches in mental health care, and encouraging further clinical research into natural compounds for managing depressive disorders.

1.6.4 Scope of study

The present study is focused on the comparative evaluation of the antidepressant potential of *Piper longum* and *Withania somniferous* extracts through a series of in vitro and in vivo experimental models. It specifically examines their ability to inhibit monoamine oxidase (MAO) activity, assesses their acute and chronic antidepressant-like effects using behavioral models such as the Forced Swim Test (FST) and Tail Suspension Test (TST), & investigates their efficacy in alleviating reserpine-induced depressive symptoms, including ptosis, catatonia, and sedation. The study is limited to preclinical investigations conducted on animal models and does not extend to clinical trials in human subjects. Furthermore, the research explores the potential synergistic antidepressant effects of *Withania somniferous* when combined with the standard antidepressant drug imipramine. The study focuses on standardized extracts of the selected plants under controlled experimental conditions, without delving into the isolation of individual phytochemical constituents or their detailed molecular mechanisms.

1.6.5 Limitation of study

1. In vitro MAO inhibition findings may not fully translate to in vivo efficacy due to differences in metabolism, bioavailability, & complex physiological interactions in living organisms.
2. Behavioral models like FST and TST primarily assess acute stress responses and may not comprehensively reflect the multifaceted nature of human depression.

3. Reserpine-induced depression models mimic only specific aspects of depressive pathology and may not capture the full clinical spectrum of depression in humans.
4. Potential synergistic effects between *Withania somniferous* and imipramine observed in animal studies may not be directly applicable to human patients without clinical validation, due to interspecies differences in drug metabolism and response.

1.7 Rationale of Work

Depression remains one of leading causes of disability worldwide, yet current pharmacological treatments are often associated with delayed therapeutic effects, undesirable side effects, & treatment resistance. These limitations highlight the urgent need for safer, faster-acting, and more effective alternatives. Traditional medicinal plants like *Piper longum* and *Withania somniferous* have been used for centuries for various health benefits, including mood enhancement and stress relief, but their antidepressant potential has not been thoroughly investigated through systematic scientific studies. *Piper longum*, known for its bioactive alkaloids, and *Withania somniferous*, recognized for its adaptogenic and neuroprotective properties, offer promising avenues for antidepressant development. This study is designed to provide a comparative evaluation of these two plants to fill critical gaps in existing research. By assessing their MAO inhibitory activities, behavioral effects in depression models, and their ability to counteract reserpine-induced depressive symptoms, the research aims to establish scientific basis for their potential therapeutic use. Furthermore, exploring the synergistic action of *Withania somniferous* with a conventional antidepressant drug like imipramine may open new perspectives on combination therapies that optimize efficacy while reducing side effects.

1.8 Hypothesis

H01: *Piper longum* and *Withania somniferous* extracts do not exhibit significant monoamine oxidase inhibitory activity compared to standard MAO inhibitors.

H02: *Piper longum* and *Withania somniferous* extracts do not demonstrate significant antidepressant-like effects in Forced Swim Test & Tail Suspension Test compared to control groups.

H03: *Piper longum* and *Withania somniferous* extracts do not significantly antagonize reserpine-induced depression symptoms (ptosis, catatonia, and sedation) in experimental animal models.

H04: The combination of *Withania somniferous* with imipramine does not produce enhanced antidepressant effects compared to individual treatments alone.

1.9 Herbal Therapeutics

The following herbal therapeutic approaches emerge:

***Piper longum* (Long Pepper)**

1. **Mechanism-Based Therapy:** *Piper longum* extract and its bioactive compound piperine function primarily through MAO inhibition with preference for MAO-B. This reversible inhibition increases monoamine neurotransmitter levels (serotonin, dopamine, and nor epinephrine) in brain, offering a mechanism similar to conventional antidepressants but with potentially fewer side effects.
2. **Rapid-Onset Intervention:** With significant effects after single-dose administration (comparable to fluoxetine), piperine could serve as a rapid-acting antidepressant option for acute depressive episodes requiring immediate intervention.
3. **Dosing Strategy:** Optimal therapeutic effects were observed at 10 mg/kg of piperine, showing dose-dependent responses without significant impact on locomotor activity, suggesting specific antidepressant effects rather than general CNS stimulation.
4. **Dual-Action Potential:** Piperine's inhibitory activity against both MAO-A & MAO- B suggests broader therapeutic applications beyond depression, potentially benefiting conditions like Parkinson's disease where dopamine metabolism is impaired.

***Withania somniferous* (Ashwagandha)**

1. **Multi-Target Therapy:** *Withania somniferous* operates through multiple mechanisms including modulation of neurotransmitter systems, HPA axis regulation, and anti-inflammatory pathways, addressing the complex pathophysiology of depression.
2. **Chronic Management Approach:** Most effective with prolonged administration (chronic dosing), *Withania somniferous* is well-suited for long-term depression management with efficacy approaching standard antidepressants (imipramine) after extended use.
3. **Adaptogenic Support:** The traditional adaptogenic properties help normalize stress responses through HPA axis modulation, providing comprehensive support for stress-related depression.
4. **Dose-Dependent Effects:** Higher dosage (40 mg/kg) demonstrated greater efficacy than lower dosage (20 mg/kg), with significant effects on reserpine-induced symptoms (ptosis, catatonia, and sedation).

Combination Approaches

1. **Synergistic Therapy:** The combination of *Withania somniferous* with imipramine showed enhanced effects, particularly in acute studies, suggesting potential benefits of combining herbal remedies with conventional antidepressants.
2. **Complementary Phytotherapy:** Theoretical potential for combining *Piper longum* and *Withania somniferous* to leverage their complementary mechanisms—rapid MAO inhibition from piperine and sustained multi-system modulation from *Withania somniferous*.
3. **Adjunctive Treatment:** These herbs may serve as adjuncts to conventional antidepressants, potentially allowing for dose reduction of synthetic drugs and minimizing their side effects.
4. **Tailored Approach:** The distinct profiles of these herbs enable personalized treatment strategies *Piper longum* for rapid relief of acute symptoms and *Withania somniferous* for long-term management and stress resilience.

CHAPTER - 2

REVIEW OF LITERATURE

2.1 Introduction

Researchers have been interested in the medicinal plants in the last several years to investigate the medicinal plant in curing different diseases and in the case of mental health conditions like depression. There is a number of plants that have been explored in terms of their therapeutic properties; *Piper longum* (long pepper) and *Withania somniferous* (ashwagandha) are some of the most promising plants due to their broad spectrum of pharmacological actions. Conventionally, both these plants have been employed in a number of systems of medicine, including Ayurveda, to treat various health disorders to treat stress, anxiety and depression. Their common uses have been extended in the recent scientific work, and now they are also studied in the potential of an antidepressant mechanism. The antidepressant activity of the plant management between *piper longum* and *withania somniferous* provides an insight into the drug mechanism and various roles that they play in mental health management. *Piper longum* is a medicinally used plant, the member of Piperaceae family, which has a millennia-long history of application. Traditionally, the use of *Piper longum* in gastrointestinal conditions and as household medicine has been attributed to it, but bioactive compounds (such as piperine) have been the cause of its therapeutic benefits. Recent research has indicated that *Piper longum* exerts its antidepressant-like effects by maintaining the quantities of brain monoamines such as serotonin, and dopamine. Research published by Mehta and Joshi (2023) indicated that the *Piper longum* contributes to the augmentation of neurotransmitters and regulates the central nervous system (CNS) through the monoaminergic systems which are involved in the path physiology of depression. This may mean that it can raise the concentration of serotonin and dopamine which may hint at it to be an alternative to usual antidepressants (those that act on the above mentioned neurotransmitters) in the context of depression.

2.2 Related Research Studies

Gupta et al. (2024) carried out a comparative study with critical comparative analysis of the antidepressant efficacy of two medicinal plants *Piper longum* (long pepper) and

Withania somniferous (Ashwagandha) in validated animal models. Behavioral paradigms- FST and TST were applied to study the anti-depressant activity. These models are frequently applied in preclinical rodent tests in testing the behavioral despair which is assumed to be reflective of antidepressant action (the reduced immobility time). In either of them, the animal is subjected to some form of inescapable stress, and its reaction is noted, especially the duration of time it spends motionless. A decrease in the duration of immobility is attributed to an improved mood and motivational state and is indicative of possible antidepressant effect. Gupta et al. It was linked to such enhanced bioactivity by presence of withanolides which is a group of steroidal lactones known to act as neuroprotectants, anxiolytic and mood uplifting. *Piper longum*, in turn, had potential effects that were the consequences of its most significant bioactive ingredient, i.e. piperine, that can alter the levels of neurotransmitters and cognitive processes in the brain. Since the individual antidepressant efficacies of both plants were shown to be significant, one of the outstanding outcomes of the research is the synergistic effect of the combination of the two plant extracts. Combined administration of the two extracts showed more reduced of the depressive behavioral than either of the extracts individually, which suggests that polyherbal remedies may be more effective therapeutically. Another significant study discovery was the safety of these herbal preparations.

Patel et al. (2024) examined the effectiveness of two of the most common traditional medicinal plants which are *Piper longum* (also known as long pepper) and *Withania somniferous* (also known as ashwagandha) in the treatment of depressive disorders. To identify antidepressant-like properties of these plants and their significance in the current psychiatric therapy, the researchers used an experimental method of comparison. Depression is a neuropsychiatric disease of multifactorial nature that afflicts millions of individuals across the globe and commercially-produced synthetic antidepressants are used extensively despite having side effects or having limited effects in certain situations. Consequently, the authors tried to find medications that would be natural so that a wider-range treatment could be achieved with less adverse side effects. The research was an integrated research study which involved the combination of behavioral tests with petrochemical analyses in order to learn the psychological and biological impacts of the plant extracts.

Mehta et al. (2023) conducted the detailed study of the molecular pathways of the action of two widely used medicinal plants *Withania somniferous* ((L.) Dunal) and *Piper longum* L. (long pepper) in terms of their antidepressant properties and offered new clues toward the development of herbal-based medications to treat depression. The researchers used the state of the art molecular biology to compare the changes in the brain tissue of animal model which was administered standard extracts of the plants, including the analysis of gene expression, the measurement of proteins and the testing of signaling pathways. The research revealed that energy drinks like *Withania somniferous* (ashwagandha) have major impact on two neurotransmitters systems: serotonergic and GABAergic both of which are relevant in mood, relaxation and anxiety.

Khan et al. (2023) This monoamine level improvement was accompanied by a stronger inhibition of monoamine oxidase (MAO) enzymes (especially MAO-A and MAO-B), which showed that a mixture of *Withania somniferus* and *Piper longum* did not only stimulate the production or release of neurotransmitters, but also their degradation. Further, micro dialysis experiments in vivo indicated that with combination treatment time dependence and persistent elevation of extracellular levels of monoamines that are directly and simultaneously monitored that are correlated with the mood. To an even greater extent than neurotransmitters, molecular studies demonstrated that the co-treatment had a positive influence on the intracellular signaling pathways underlying neuroplasticity, cellular repair and stress resilience processes that are often disrupted during depression. In particular, brain-derived neurotrophic factor (BDNF) and other neuroprotective protein signaling were not regulated, which offered the potential of the combination, which would retain long-term brain health and control over emotion. It is hypothesized in the study that the monoamine-modulating, MAO-inhibiting, and neurotropic-signaling potential of *Withania somniferus* and *Piper longum* could be the interacting forces which exist as a result of antidepressant effects of the 2-herb combination. The outcome of these findings provided a bright perspective to the possibility of incorporating these polyherbal formulations into the next generation antidepressant treatment policy, especially among the patients who do not embrace the traditional pharmaceutical agents in the treatment process based on the reduced side effects and increased acceptability.

Thapa et al. (2022) This finding is noteworthy in both terms of coming up with standardized tissue culture protocol to multiply mass as well as the production of secondary metabolites of *Piper longum*. More to the point, the research has not been limited to the effects of hormones, but also included the researches on the impact of different environmental signals, i.e. light conditions and nutrient content of the medium, on the rate and quality of the callus formation and proliferation. These must have been crucial parameters influencing callus properties in growth and fine manipulation of the state of culture would lead to improved performance. In addition, phytochemical analysis of the callus was done which also established the presence of major alkaloids, piperlongumine, piperlonguminine and piperlongumine. Although they had lower yields relative to those produced in nature, their detection and indication of the in vitro culture as a substitute source of these pharmacologically useful metabolites was made. It is worth noting that the study indicated that optimization of the in vitro culture which may be through elicitation by introducing certain stressors to trigger the production of bioactive, could significantly increase the production of these metabolites.

Saini et al. (2022) The study presented here did not investigate the specific effects of *Piper longum* or *Withania somniferus*, but nevertheless provided insights into the overall mechanisms by which the endophytes that are associated with plants can regulate the therapeutic effect of medicinal plants. Of particular interest was the enzyme-inhibition properties of the extract which in turn, in tests interestingly inhibited acetyl cholinesterase (an enzyme required to break down acetylcholine, which in turn can be attacked by medicines in neurological and neurodegenerative diseases, including some antidepressants and Alzheimer drugs) and prostaglandin E2 (cause of swelling and pain). The extract also had a strong antioxidant activity as measured by distinct assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2, 2'-azino -bis(3-ethylbenzothiazoline-6-sulfonic acid)) and Hydrogen peroxide scavenging assays among others. Combined, these assays revealed the capacity of the extract to scavenge free radicals and thus it becomes a viable neuroprotective compound in oxidative stress- related disease, such as depression, Parkinson disease, and other cognitive disorders.

Islam et al. (2022) The inclusion of *Piper longum* or *Withania somniferus* in their planned work was not part of the purpose of the study; instead, it provided an effective comparative groundwork and methodological curiosity in the screening of neuropharmacological profile of plants. There were several well described models of behavior that were employed in order to test the activity of the CNS, specifically: Open field test, whole cross test and Elevated plus maze). The results of these behavioral tests were clear demonstration of strong anxiolytic and sedative effect of *Lannea coromandelica* extract, and can be taken as the viewable agent of reducing the symptoms of anxiety and perhaps of mental health- especially in a situation where anxiety and depression are comorbid.

Rahman et al. (2021) gave a full insight on ethnomedicinal use of antidiabetic plants in Bangladesh not only in management of diabetes, but also its potentials at large, which is primarily on mental health. The review was stringent in documenting the various species of plants that the rural people found useful in the management of blood sugar levels, how the plants were prepared to be ingested/utilized (i.e. decoctions, infusion, powders or raw extracts) or how the plants were applied (orally or topically) and the perceived effectiveness of the plants against the traditional wisdom. Importantly, another sub-group of these antidiabetic plants also known to have psychoactive or neuroprotective effects also received attention by the authors, which highlights the questionably understated, yet vital, fact of the relationship between metabolic disorders and mental health. It is an interesting discovery because it suggests that even well before -metering devices and the current medical understanding of health and wellness in general, the interconnection between metabolism health and psychic wellness was felt by the so-called medicine men, and that these individuals learned to treat two conditions using plants common to the area.

Jobi et al. (2020) performed a comparison of the antioxidant properties of the fruits and leaves of the two species of Piper, *Piper longum* and *Piper nigrum*. Following the potential neuroprotective effects of these plants especially on depression and other neuropsychiatric diseases, research was to be conducted to provide a comparative understanding of the relative antioxidant properties of the different plant extracts. There was minimal difference between leaves and fruit extracts of the same species in a number of antioxidant-environmental assays (including the 2, 2-diphenyl-1-

picrylhydrazyl (DPPH) radical scavenging as well as the 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)). Interestingly, fruit extracts of *Piper longum* had the best DPPH radical-scavenging activity (the greatest radical scavenging effect) in comparison with the other samples examined.

Franco et al. (2020) researched the anti-diabetic effect of *Syzygium cumini* (Jamun) leaves compared to their non-hemolytic effects and the inhibitory effect of oxidation, glycation, inflammation and their activity against digestive enzymes. Even though other conventional herbal medicines were not researched in this study i.e. *Piper longum* (long pepper) or *Withania sonnifera* (ashwagandha), the findings of the two as reported in this study were representative of the universal curative potential of the plants that express more than a single mode of action and interest-getting, eventually was very significant in enhancing and building knowledge base of compound medicinal uses of herbs. The researchers also fractionated extracts so as to isolate the most active constituents, and discovered that the highest levels of flavonoid were found in the fractions that showed the highest effects in all of the biological assays. This methodology gave a systematic way of anticipating the multi-target effects of the medicinal plant, and indicated that it was highly essential to determine the active agents of the plant extract. The findings in this paper find particular relevance to the study of medicinal plants including those that are used in the management of multifactorial conditions like diabetes and depression which is a condition where several underlying pathological mechanisms occur, and many. These characteristics of *S. cumini* leaves, such as the multiple positive activities, the absence of toxicity, accentuate the potential of the plant in terms of generating many-sided therapeutic treatment of chronic diseases and can be used as a basis in additional research on the therapeutic utilization of the plant.

Patel et al. (2020) provides a comprehensive review on the therapeutic applications of two traditional Indian ayurvedic drugs which include: *Withania somniferus* (ashwagandha) and *Piper longum* (long pepper) about their mechanistic actions in the treatment of depression. In this regard, scientists searched to connect the gap between the prior knowledge and the contemporary pharmacology in the production of the most recent findings on the molecular and cellular basis of the antidepressant effect of plants. The examined study was meant to investigate pre-clinical data to establish

possible mechanisms of how *Withania sonnifera* might treat depressive symptoms. The plant is known to possess an adaptogenic behavior, and Patel et al. (2020) emphasized the involvement of the plant in the processes of GABA-ergic neurotransmission regulation, which is essential in the central nervous system sedation. The authors indicated that a potential role of *Withania sonnifera* is the up-regulation of BDNF which is a protein that plays a crucial role in neuroplasticity and neuronal survival. The latter party highlighted that the plant would be able to normalize the sensitization of HPA which is a common characteristic of depression and also associated with chronic stress. These data point to the multi-target effects of the plant that can also alter the neurotransmitter systems, neuroendocrine functioning and neuroplasticity, the three systems that are confounded in depression.

Verma et al. (2019) The results of the current research indicate a significant reduction in immobility time by both *Withania sonnifera* and *Piper longum* during the forced swim test as well as the tail suspension test like the conventional antidepressant imipramine that indicates antidepressant activity. On LHE where both the plant extracts also considerably reduced the reductions in the escape failures implying that they can reverse depression-like behaviors. The dose-response appraisals that further describe the most suitable doses indicated that both plants had a dose- dependent effect and the highest doses produced the best antidepressant-like effect. Even though experiments with behavioral effects were conducted, petrochemical studies were also carried out by the authors to clarify the action mechanism. Their findings revealed that both *Withania sonnifera* and *Piper longum* had the ability of increasing the monoamine concentration of the brain though with varying regional selectivity and also the impact on a specific transmitter such as serotonin, dopamine and nor epinephrine. The antioxidant properties of these plants were also researched as they could be the cause of their antidepressant effects. The amelioration of markers of oxidative stress was found to correlate with the improvement of the behavior, which suggested that the antioxidant activity of the plants was a factor in their pharmacological effect. The latter compound also substantially counteracted the radiations effects so that it reached corticosterone level in the irradiated dogs in the process of usage.

Zhang et al. (2018) have provided a review of the techniques of isolation and extraction of active compounds of medicinal plants. They have also extensively discussed the ways of extraction i.e. maceration, percolation, Soxhlet extraction and even the modern techniques like ultrasound assisted extraction, microwave assisted extraction, supercritical fluid extraction (SFE). The authors reviewed the efficiency, selectivity, and usefulness of these methods to the extraction activities of the bioactive compounds of plants. They emphasized that there must be proper extraction techniques based on the physicochemical characteristics of the targeted compounds and also on the characteristics of the plant materials. The review also presented the information about post-extraction processes, including chromatographic analysis, separation and purification procedures, which are vital in pharmaceutical application, where the high level of purity is necessary to obtain the bioactive molecules.

Sood et al. (2018) In an animal study done on stroke induced by middle cerebral artery occlusion (MCAO), Sood et al. (2018) investigated the impact that *Withania sonnifera* (also known as Indian ginseng) has on oxidative stress and mitochondrial dysfunction. The research was postulated based on previous studies which indicated significant increase in reactive oxygen species (ROS) levels between 3 and 23 days after ischemic stroke, and thus the ability of *Withania sonnifera* to suppress the production of ROS may be the cause of the infarct volume reduction. This inhibition is likely to be the reason behind the reduction of malondialdehyde (MDA), which is an indicator of oxidative stress, in the subcortical area following MCAO. They concluded that the administration of *Withania sonnifera* had significant effects of reducing the oxidative stress markers and improving the mitochondrial activity of the experiments of stroke induced parts of the brain in animals. The authors indicated that *Withania sonnifera* and more precisely, its phytochemicals including withanolides that were also identified in *Withania sonnifera* had a number of active bioactive compounds that were involved in neuroprotection through antioxidant mechanisms. The treatment of the animals was identified to have had considerable improvement in behavioral parameters and neurological deficits meaning *Withania sonnifera* can be considered a potential phytomedicine in the treatment of stroke. Their findings affirmed the use of this medical plant in neurological disorders and that could be regarded as useful drug to prevent and treat stroke.

Venkatachalapathi et al. (2018) surveyed the Irula tribes, population of which dwells in the Southern Western Ghats, in the valley of Walayar in India. This large scale ethnobotanical survey recorded the traditional medicinal plants that reminded of plants that were utilised to treat different diseases in these tribes and ecosystems. Researchers identified numerous species with therapeutic properties that Irina tribes apply in their traditional medicine. The name of all medicinal plants, part used, mode of preparation, route of administration and specific mode of treatment were thoroughly recorded. Most of these plants were recorded to be employed as medication against these diseases such as fever, respiratory, digestive disorders, skin diseases, and inflammation. The researchers pointed out the necessity to document and preserve this ancient testimony of the ancient ancestral knowledge that is intellectual property of aboriginal people and could hold the precious information about current methods of drug discovery.

Yadav (2018) performed review of the two drugs, *Piper longum* and *Withania sonnifera*, in treatment of major depressive disorder. The extensive research estimated safety profiles, efficacy, and antidepressant mechanisms of these two widely used medicinal herbs in traditional medicine. The authors have examined clinical and preclinical evidence that was indicating the use of these herbs as a treatment of symptoms of depression. The review also states that the bioactive chemicals found in both plants can have multiple modes of action associated with the pathophysiology and etiology of depression including anti-inflammatory effects, hypothalamic-pituitary- adrenal axis regulation, and neurotransmitter system regulation. Piperine in *Piper longum* and withanolides in *Withania sonnifera* were identified as the main substances that were responsible of the antidepressant effects. The authors observed that joint preparations of these plants could provide synergistic effects based on a complementary action. The review has highlighted the importance of these traditional medicinal plants as alternatives or additions to the conventional antidepressant therapies especially in patients who want natural forms of therapy.

Rao (2017) has presented a neuropharmacological comparison of *Withania sonnifera* and *Piper longum* as antidepressants. Their paper has conducted a systematic evaluation of the impact of these medicinal plants on the different neurotransmitter systems that are involved in depression. The study established that both the plants had

notable antidepressant-like actions in the experimental models albeit with slightly dissimilar neurochemical pathways. The effects of *Withania sonnifera* were more on serotonergic and GABAergic systems whereas *Piper longum* had greater effects on dopaminergic and noradrenergic systems. The authors noted that the interdependent effects of extracts of both plants on antidepressants were stronger than when any one plant was used, hence there might be synergetic effects. The research presented neurochemical justifications of the historical use of these plants as a combination in mood disorders and their possible application as the origin of new antidepressant compounds. The researchers highlighted the importance of clinical research that should be used to further confirm these findings in human depression subjects.

Kala (2017) conducted GC -MS analysis of the physiologically active compounds in the *Canthium parviflorum* Lam callus and leaf extracts. The objective of the research was to discover and compare phytochemical profiles of in vitro callus cultures and leaves of this medicinal plant that grow in the wild. The gas chromatography-mass spectrometry reported that in the extracts of the leaf and callus, many bioactive compounds were present, such as alkaloids, terpenoids, phenolic compounds, and fatty acids. Remarkably, their study confirmed the conventional therapeutic uses of *Canthium parviflorum* and found the use of callus culture would prove viable biotechnological tool that would enable the production of bioactive chemicals in the plant in a sustainable production.

Shah et al. (2017) documented Khushab, Soon Valley of Pakistan report of ethnomedical use of plants in treatment of malaria. During the field research, many different plant species that, in the past, have been utilized by the local communities in treating malaria and its symptoms were discovered. The extensive information on such antimalarial plants, including botanical name, common names, parts, method of preparation and route of administration was recorded in study. The scientists found that many of the traditionally used plants had large amounts of bioactive chemicals that had the antimalarial effect had been established in previous research and that the traditional uses of these plants were logically justified. The authors discovered a range of interesting plant species which require further pharmacological studies in the event that new antimalarial drugs could be developed. As the study concludes,

documentation of this traditional knowledge as a valuable cultural heritage as well as leads to is important.

Mohan et al. (2017) has surveyed ethnomedicinal plants in the Kawal Wildlife Sanctuary of Telangana in India. Their extensive field study documented great variety of medicinal plants that were used by the indigenous inhabitants residing and surrounding this protected area. Researchers recorded many specifics about plant species with medicinal value including their slang names, their used parts, methods of preparation, and specific use in various medical conditions. Authors emphasized the importance of preserving this historic knowledge that can be utilized in the field of modern medicine and in cultural heritage. Research also focused on conservation problems the medicinal plants resources in the area have to deal with due to loss of habitat and poor harvesting practices.

Li et al. (2016) Withaferin a Anti-Cancer Mechanisms: Lipid Metabolism in Skin Carcinogenesis This is an original study into the effects of withaferin A on the development of skin cancer, which examines the relationship between the molecule and its impact on cancer formation in terms of its molecular mechanisms of action. The study targeted withaferin A, a steroidal lactone extracted out of *Withania sonnifera* (also called Ashwagandha), and its particular action in acetyl-CoA carboxylase 1 (ACC1) and resultant tumor development in a skin cancer experimental. The two-stage skin carcinogenesis protocol adopted by the researchers was well established, probably with DMBA (7,12-dimethylbenz[a]anthracene) as an initiator and repeated applications of TPA (12-O-tetradecanoylphorbol-13-acetate) as a promoter, a model that is much more similar to the human skin cancer development process. This experimental platform offers a valid channel of testing potential chemopreventive agents based on the attempt to mimic the initiation and promotion stages of carcinogenesis. The reported decrease in the recurrence and multiplicity of tumors after withaferin, a administration illustrates the superb effectiveness of the compound in cancer growth prevention. The number of tumors per animal (multiplicity) and percentage of animals developing tumors (incidence) was much lower in treated animals which implied protection at the initiation and promotion phases of carcinogenesis. This dual protection indicates that withaferin A has several complementary effects as an inhibitor of tumor formation. The most novel conclusion

of the study is focused on the fact that withaferin A has the capability of inhibiting the upregulation of ACC1 in tumor tissues. ACC 1 is involved in the rate-limiting step in the synthesis of fatty acids de novo, which is the transformation of acetyl-CoA to malonyl-CoA. The increase in lipid metabolism is a characteristic feature of cancer cells that need to provide intensive growth and membrane renewal.

Wu et al. (2016) The novelty of the current study on the *Salvia miltiorrhiza* callus cultures can be described as an important step in the development of the pharmaceutical biotechnology field, as it has managed to integrate the knowledge of the traditional medicinal plants and the new methods of production to come up with potential cancer treatment products. The study was directly concerned with the optimization of the synthesis of two useful phenolic derivatives, namely: rosmarinic acid and salvianolic acid B, and their selective cytotoxicity towards acute lymphoblastic leukemia cells. *Salvia miltiorrhiza* (also referred to as red sage or Danshen) has a long history of use as a traditional Chinese medicine in the treatment of cardiovascular and cerebrovascular diseases. This study however investigated its under-researched anticancer properties, especially those that might be synthesized by biotechnological approaches as opposed to the traditional cultivation. This study makes use of the plant tissue culture methodology that has several benefits over the conventional cultivation techniques, such as annual-round production regardless of geographical and seasonal limitations, strict regulation of the growth environment, and avoidance of environmental contamination. The researchers developed callus cultures which are undifferentiated masses of plant cells of *Salvia miltiorrhiza* presumably by culturing leaf or stem segments in special media with special plant growth regulators. They attained very high levels of production of the target compounds by a systematic optimization of culture parameters such as nutrient composition, concentrations of plant growth regulator, light conditions, temperature and elicitation strategies to an extent much higher than the parent plant material. It is most likely that this optimization process entailed an experiment of different combinations of auxins and cytokinins which are the main hormones of the plant that control callus induction and callus maintenance. The high compound yield which is dramatically increased is a great breakthrough in plant biotechnology.

Gupta (2015) Comparative Antidepressant Mechanisms of *Piper longum* and

Withania sonnifera, this is a significant step forward in the research on the antidepressant potential of traditional medicinal plants because it uses a rigorous scientific method in its study. The authors utilized various complementary behavioral models to comprehensively assess the impact of these plants on depression like behaviors and provided multidimensional support to the therapeutic potential of these plants and established unique mechanistic signatures that can guide their optimal clinical implementations. The experimental design must have required standardized extracts that are given to various doses and at different times so that dose-response and time-course analyses, which are very important to pharmacological characterization, can be done. The researchers were able to capture various aspects of the depression-like behavior by using several behavioral models such as forced swim test (depression behavioral despair), tail suspension test (motivational deficit measures) and learned helplessness paradigms (measures of cognitive aspects of depression). Such a multi-model model can be especially useful since each of the tests can measure more or less different behavioral and neurobiological variables of depression, making it a comprehensive evaluation of antidepressant potential than the individual tests would have provided. The recorded variation in potency and time effect in *Piper longum* and *Withania sonnifera* in the behavioral parameters depict significant variance in the pharmacological characteristics of the two plants. *Withania sonnifera* could have had slower onset yet longer lasting effects in line with its adaptogenic status whereas *Piper longum* could have had faster action in line with its direct neurochemical action of its bioactive alkaloids.

Shah et al. (2015) The novel research examining Ashwagandha (*Withania sonnifera*) leaf extracts mixtures is a major step towards uncovering the neurobiological evidence of the impact of this traditional medicine plant on the brain. It was the first study to examine specific extract combinations instead of individual compounds or crude extracts because the therapeutic properties of the plant are probably due to synergistic interactions between several bioactive compounds that act by complementary mechanisms. The researchers have used advanced in vitro models to assess two different yet related neurobiological events namely neuroprotection against oxidative stress and neuronal differentiation. In the case of oxidative stress measurements, they probably used cultured neuronal cells or brain-derived cell lines, which were subjected to diverse stressors- which may be hydrogen peroxide,

glutamate excitotoxicity or amyloid-beta peptides- representing the different pathology mechanisms with regard to neurodegenerative disorders. The reported defense of Ashwagandha extracts in these conditions implies outstanding flexibility in the prevention of various types of neuronal damage. The process of neuronal differentiation promoted by the extract can be discussed as probably the most innovative finding of this study. The researchers used probably the neural stem cells or neuronal precursor cells, and they found that the Ashwagandha treatment was very effective in promoting the functioning of the cells by differentiating them into the mature neurons.

Sharma et al. (2015) The ethnobotanical survey study in the National Highway 5, Himachal Pradesh, Solan and Shimla is also an important addition to the knowledge of traditional medicinal plant resources in transitional ecological zones. The region is a key biodiversity corridor in the western Himalayas despite the growing human influence on it through highway construction and urbanization. The researchers were able to systematically record the high number of plant species that are medicinally valuable and are still used by the local people to treat different ailments. They detailed botanical identifications, local dialects used the names, parts of the plant that they used, traditional methods of preparation, and the therapeutic use. This careful record keeping ensures that important ethnomedicinal information is retained that would otherwise be lost as the traditional traditions are eroded. The authors of the study have found that plant species along the highway strip are substantially resolute to environmental stressors, one of which is vehicular pollution.

Raghavan (2015) This is an innovative study that sheds light on the molecular pathways via which [*Withania sonnifera*] (also known as Ashwagandha) produces neuroprotective effects in a state of ischemic stroke. The research is a major leap in the knowledge on the mechanism of action of this ancient medicinal herb that has been worshiped in Ayurvedic medicine over the centuries and its role in alleviating neuronal injury in cerebral ischemia, which is a lack of blood flow in the brain tissue which causes complex cell death pathways. The researchers have shown that treatment with *Withania sonnifera* extract led to a significant decrease in the largest infarct volume and a positive outcome in the recovery of neurological function in stroke models. Such macroscopic benefits were linked to the maintenance of neuron

architecture and the loss of fewer cells in the penumbra, which is the most vulnerable and potentially salvageable tissue in the periphery of the core infarct.

Sachan et al. (2015) This is one of the pioneering studies focusing on the association between agro-climatic and ethno-medicinal plant use in Uttar Pradesh that provides some important insights into the role of ecological factors in influencing the traditions of knowledge in medicine. A state with an impressive geographical diversity, Uttar Pradesh, between the Himalayan foothills in the north and the Gangetic plains in the central and southern areas, is a perfect place to examine the impact of environmental changes on the distribution of medicinal plants in the area and the conventional activities. The researchers used an extensive ecological method of ethnobotanical reporting which used a division of the state into specific agro-climatic regions such as the Terai region, western plains, central plains, eastern plains, Vindhyan plateau, and Bundelkhand region. All the zones have their own unique mixtures of the temperature patterns, rainfall distribution, the humidity and soil types and topographical features of the land that are combined to form unique plant habitats. The approach to methodology enabled the researchers to examine the relationship between these environmental parameters and medicinal plant diversity, abundance, and traditional patterns of utilization. The research paper recorded that a great variety of medicinal flora were found to be adapted to certain ecological niches in these zones.

Subedi et al. (2014) The study about eight medicinal plants in the western Nepal area is an important addition to the ethnopharmacological literature between traditional medicine and modern scientific research. The paper thoroughly analyzed the connection between centuries-old therapeutic use and biochemical characteristics, namely, antioxidant activity and phenolic and flavonoid constituents. The authors used several complimentary antioxidant assay systems to fully test the abilities of the plants to scavenge free radicals. These were probably DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay, ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging test, ferric reducing antioxidant power (FRAP) and perhaps oxygen radical absorbance capacity (ORAC) measurements. The methodological diversity enhances the validity of the findings because it takes into consideration different mechanisms of antioxidant activity. The total phenolic content was presumably determined through Folin-Ciocalteu method with the results in gallic acid

equivalents, and the flavonoid content was probably determined through aluminum chloride colorimetric method with the results in quercetin or rutin equivalents. Such standardized methods permit significant comparisons of similar studies conducted in other areas. The analysis showed that there was a tremendous difference in antioxidant potential of the eight medicinal plants, and a direct relationship existed between the antioxidant capacity and the concentration of phenolic and flavonoid compounds.

Singh et al. (2014) The ethnobotanical survey in the Vindhya area, east Uttar Pradesh, is an essential record of existing cultural heritage and information on medicinal plants. The study throws light on the long-lasting association between the local community and the surrounding botanical landscape especially when it comes to provision of primary healthcare services within the locality that has little access to the modern medical facilities. The Vindhya range, with various topography and micro climates, boasts of a wide variety of medicinal flora which have been used over the centuries by indigenous curers, home makers and traditional caregivers. The records had detailed botanical names of the medicinal plants, local vernacular names (typically describing the properties or application), and parts of the plant used medicinally; root, leaf, bark, flower, fruit or seed. Precisely, the preparation procedures were documented in the research; generally, they encompassed processes like decoction (boiling the plant material in water), infusion, preparation of paste, powder formulation, or solvents extraction with use of local solvents, such as water, milk, or oils.

Vaidyanathan et al. (2014) The ethnomedicinal research performed among the Malayali tribal of the Vellakadai hamlet in the Shervaroy range of the Tamil Nadu Eastern Ghats forms an important part in the area of understanding the indigenous knowledge system and how this would be helpful in the current medical scenario. This study sheds light on the high-level botanical knowledge of one of the most ancient tribal groups in India, who have had a close association with the forest ecosystem, and, over many generations, a complex pharmacopoeia based on the local flora has been developed. The Malayali tribe that was named after malai, which translates to hill, has long been living in the higher grounds of the Eastern Ghats where they cannot be easily connected to the outside world, thus, maintaining their

cultural practice and traditional knowledge. This seclusion, although proving to be a challenge to the economic growth, has unwillingly contributed towards preserving the rich ethnomedical practices that would otherwise have been destroyed by the massive interaction with the mainstream medical systems. The knowledge that is documented is not just an empirical observation but rather a sophisticated knowledge of the properties of plants, seasonal changes, harvesting methods, and therapeutic use that has been perfected over generations of experience. The methodical records made by the scientists recorded botanical names in standard taxonomic nomenclature and local colloquial names which in many cases give linguistic indications of the characteristics or utilization of the plants.

Shaikh et al. (2014) carried out a comprehensive ethnobotanical survey of folk medicinal plants in use by the villagers in the Nanded district of Maharashtra, India. Their study reported on the deep traditional knowledge on plant-based remedies which had been maintained across the generations in the region. The research design was a field visit research design where semi-structured interviews were carried out on 20 villages among the 78 local healers and elderly members of society, who possessed specialized information about medicinal plants. The researchers managed to determine 126 species of plants used in traditional healing practices which are active, this figure represents 58 families. They have carefully documented local vernacular names, parts used, preparation modes and dosages and specific therapeutic uses of every plant. Their results were that Fabaceae, Euphorbiaceae, and Asteraceae were the most commonly used plants in the local medicine. The paper has reported that the most common part of a plant to use was the leaf (41%), then roots (22%), and fruit (17%), and the most common route of administration was by mouth using decoctions.

Sarkhel (2014) This expert study covered a field of paramount concern in the rural setting where snakebite is a major population issue and where timely access to traditional antivenom treatment is usually scarce. The researcher used a systematic research methodology which included field surveys of 15 villages and organized interviews on 45 traditional snake bite healers in the area referred to as Ojhas in order to map the plants being used and their mode of application. In this way, Sarkhel was able to recognize 24 species of plants of 17 families that are specifically used in snakebite treatment by local practitioners. The paper has carefully recorded the modes

of preparation, the route of administration, dosages, and certain symptoms that each remedy is aimed at. The scholar noted that cures were normally provided in the form of pastes which were direct applied to bite wounds, oral decoctions or both methods.

Prakash et al. (2014) is a pioneering research done to test the neuroprotective capability of *Withania sonnifera* in the models of Parkinson disease. The purpose of the research was to identify the molecular processes in which this traditional medicinal plant has its effects on the benefits it has been reported to cause in neurodegenerative disorders. The researchers tested the effects of *Withania sonnifera* extracts on dopaminergic neurons- the key cell type that is affected in Parkinson disease using a complex method, which involved both in vitro cellular models and in vivo animal work. The paper showed that the standardized aqueous extracts of *Withania sonnifera* had a pronounced alleviating effect on the parkinsonian phenotypes by blocking various apoptotic processes in the dopaminergic neurons. The researchers used advanced molecular biology enhance methods to find out that the extract silenced the expression of pro-apoptotic proteins such as Bax, caspase-3, and caspase-9 and increased anti- apoptotic proteins such as Bcl-2. Immunohistochemical studies of brain tissues in treated animals revealed that at substantia nigra and striatum- the areas in the brain that are critical in Parkinson disease, tyrosine hydroxylase positive neurons were preserved.

Pingali et al. (2014) was done to determine the impact of standardized aqueous extract of *Withania sonnifera* on cognitive and psychomotor tests performance in healthy human subjects. This study is a major step towards the scientific validity of conventional assertions of the cognitive-enhancing ability of *Withania sonnifera*, since it used reliably-tested psychometric scales in a controlled human trial research design. The researchers enrolled 20 healthy male participants with the age group of 20-35 years in this randomized, double-blind, placebo-controlled crossover study. The randomized controlled trial was done on subjects who were given 250 mg of standardized *Withania sonnifera* extract or placebo twice a day during 14 days with a 14-day washout duration between the sessions. Cognitive and psychomotor performance were measured with the help of a battery of tests such as the digit symbol substitution test (DSST), simple reaction time (SRT), choice discrimination test, finger tapping test, and card sorting test all measured measures that had been validated

before to measure different cognitive areas such as processing speed, attention, sensorimotor and executive functions. The findings proved statistically significant changes on reaction time, card sorting performance and digit symbol substitution scores after the administration of *Withania sonnifera* over placebo.

Nabi et al. (2013) was aimed at the thorough research of the antidiabetic and antihyperlipidemic properties of the aqueous extract of *Piper longum* root in streptozotocin-induced diabetic rats. Their study came at a time when there was a significant demand to have evidence-based administration of the traditional antidiabetic remedies, especially with the increased prevalence of diabetes mellitus in the world. The researchers used a properly designed protocol of the experiment, which involved the induction of diabetes in the Wistar rats by injection of streptozotocin, and *Piper longum* root aqueous extract was administered to them (200 mg/kg and 400mg/kg body weight) over a period of 30 days. The parameters that the researchers followed (such as fasting blood glucose, glycosylated hemoglobin, plasma insulin, lipid profile, and hepatic glycogen content) allowed the researchers to determine comprehensively the antidiabetic activities of the extract. They found the dose-dependent hypoglycemic activity and the high dose proved as effective as glibenclamide which is the standard antidiabetic drug that was used as positive control.

Bucchini et al. (2013) developed a unique study that aimed at exploring the total polyphenol content and antifungal and antioxidant activity of in vitro callus cultures *Inula crithmoides*. Their study is a great contribution towards the diversity of plant biotechnology in treatment of medicinal plants, and overcomes the problem of sustainable synthesis of bioactive compounds of useful medicinal plants. The researchers determined the callus cultures of *Inula crithmoides* by means of the leaf explants of the plant cultivated on optimized compositions of the plant growth regulators and observed their growth characteristics through a series of subsequent subcultures. Comprehensive phytochemical profiling of the extracts of the callus was done with respect to total polyphenol content and then the extracts were analyzed in terms of their biological activity.

Adhikari et al. (2013) arranged a comparative examination of the antimicrobial

effect of the chemical compounds in vivo root and in vitro callus of *Withania sonnifera* (L.) Dunal. Their study deals with the burning issue of the sustainable production of bioactive compounds of medicinal vegetation without the loss of natural populations. The authors have raised callus cultures on MS medium amended with various ratios of plant growth regulators using the leaf explants of *Withania sonnifera*, which maximized the biomass growth. They used several chromatographic and spectroscopic methods to analyze root extracts of field-grown plants and callus extracts in detail. The antimicrobial screening was done on eight bacterial strains and four fungi species based on conventional techniques such as agar well diffusion and minimum inhibitory concentration determination. Their results indicated that there was a drastic variation in chemical composition of in vivo roots to in vitro callus where withanolides, especially withaferin A and withaferin D, were found to be more in roots grown in the field. Interestingly, in spite of such differences in composition, the callus extracts showed similar antimicrobial activity in relation to root extracts against a variety of test organisms and in particular, strong activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans*.

Singh (2012) is significant as the author documented less known ethnomedicinal applications of the plants in the Odisha state, Sundargarh, Mayurbhanj, Angul, and Balangir districts of India. This study is valuable because it helps in maintaining the old knowledge that may be lost in the process but also finding likely plant candidates that may be used in future pharmacological research. The author used field survey techniques such as interviewing of traditional healers, village elders and local practitioners to find out the information on medicinal plants and how they were traditionally used in these areas. The documentation process involved the collection of plant samples, taxonomic identification and the clear description of the preparation procedure, dosage and therapeutic use of each plant. The results were a revelation of a wide array of medicinal plants employed by the indigenous people in these districts in the treatment of all types of ailments with many of them not being recorded in scientific literature.

Uddin et al. (2012) have made a systematic review of phytochemical and pharmacological profile of *Withania sonnifera* Dunal that summarizes the available information regarding this valuable medicinal plant. Their article gives a very

comprehensive review of the chemical contents, traditional applications, and evidence-based treatment interventions of *Withania sonnifera*, thus, it can serve as a resourceful source to researchers in the natural products and ethnopharmacology field. The literature review was done in a systematic way and both included ancient literature explaining traditional use of the plant and modern scientific literature that reported some experimental and clinical results of this plant. They assigned the information as per chemical composition, pharmacological activities, toxicity profiles and clinical application with the aim of providing a comprehensive picture of the medicinal value of the plant.

Kumar et al. (2011) carried out a comparative analysis of adaptogenic and antidepressant effects of *Withania sonnifera* and *Piper longum* in laboratory animals and this study offers important knowledge on the dosage potential of these traditional medicinal plants. Their study adopted an extensive experimental methodology in which they have used well-established animal models to determine both adaptogenic effects (capacity to enhance non-specific resistance to stress) and antidepressant effects. The research involved different behavioral paradigms of forced swim test, tail suspension test, open field and elevated plus maze with biochemical analyses to determine the effects of these plant extracts in isolation and during the integration of these extracts. The researchers made standard extracts of the two plants and then subjected them to experimental animals at varying dosages to determine dose-response relationships. Vehicles or standard drugs (fluoxetine to induce antidepressant activity and diazepam to induce anxiolytic effects) were used to compare the control groups.

Bhatnagar et al. (2011) on the antidepressant-like properties of mixtures of *Withania sonnifera* and *Piper longum* offered a detailed study of the effect of the mixtures on the behavioral and neurochemical characterization. The study is an important contribution to the body of scientific knowledge on Ayurvedic medicine herbal combinations in the treatment of mental illnesses. The researchers used a systematic strategy in the assessment of the effectiveness of these plant extracts as a whole, as individuals and in combination to assess the effectiveness using the already available animal models of depression. Various behavioral paradigms such as the forced swim test, tail suspension test, and the learned helplessness models were used as the

methodology in the study, and they are generally accepted behavioral paradigms in the screening of antidepressant compounds. Besides the behavioral testing, the researchers provided elaborate neurochemical analyses in order to establish the possible mechanisms of the observed antidepressant-like effects. The experimental design involved a set of treatment groups that were given a varying amount of the *Withania sonnifera* extract, *Piper longum* extract, both together in various ratios, and the conventional antidepressant drugs to make a comparison. The findings showed that the individual plant extracts had a great dose-dependent antidepressant-like behavior on all the behavioral models. Nevertheless, the most fascinating was the synergistic effect on the effect of the two extracts when they were used together, especially at particular proportions.

2.3 Research Gap

Even though the evidence at hand is pointing towards the antidepressant activity of the *Piper longum* and *withania somniferous* plants there are still numerous gaps in the comparison of these two plants particularly in terms of their mechanisms of action, effective doses and chronicity. The independent antidepressant-like actions of the two plants in different models are well reported but the molecular and neurobiological outcomes of these plants when used concomitantly are not well understood. Their synergy is only studied in early stages and additional research is required to explain how the various pharmacological effects of them can be combined to enhance their efficacy with respect to therapy. Moreover, although the anti-depressants properties of *Piper longum* and *Withania somniferous* have been reported in animal systems, little exists in human research. The short-term effects are commonly reported in the literature and thus there is no evidence on the safety and effectiveness of long-term effects. Moreover, when using *Piper longum* and *Withania somniferous* with standard anti-depressants, it is also required to consider and seek the optimal dosing schedules, and the potential possibility of the drug to interact with other anti-depressants in use. And appraisal of the effects of these biological agents on antidepressants has no standardized norm. Diversity of experimental models is utilized in most of the existing studies and it makes the comparison of studies difficult. There is the need to develop sound evidence to support their use in clinical practice by means of

standardized protocols and controlled clinical trials. Those gaps should be addressed to investigate the therapeutic potential of *Piper longum* and *Withania somniferous* as an antidepressant.

CHAPTER - 3

MATERIALS AND METHODS

The chapter outlines different systematic experiments that were used in comparing two medicinal plants; *Piper longum* and *Withania somniferous* in their antidepressant effect. The chapter describes the procedures involved in collection of plant material and its authentication, extraction procedures of both plants, qualitative and quantitative screening of phytochemicals and isolation of bioactive compounds and characterization of the same by use of spectroscopic methods. It also talks about animal models of preclinical screens, in vitro assays i.e. MAO inhibition, in vivo behavioral tests: Forced Swim Test, Tail Suspension Test. chapter also includes the preparation of the test doses, and the layout of the experiment which includes 6 groups of animals whose methods are used to conduct the biochemical assays as well as ethical considerations followed during the study.

3.1 Collection and Authentication of Plant Material

3.1.1 Source and Collection

Dried *Piper longum* berries were obtained with care in a well-known herbal pharmacy in Sagar, Madhya Pradesh, India. This vendor has a long history of providing high-quality plant materials that are suitable in the pharmacological investigation and thus this vendor was selected. The decision of buying the dried fruits in a well-known herbal pharmacy which strictly follows the quality control measures and regulations of the place of origin and preservation of the plant materials was not accidental. When buying the dried fruits, they were stored at 4 ° C in darkness to ensure stability, as well as protect the bioactive contents against degradation. This storage method ensured that the *Piper longum* fruits were not contaminated or damaged chemically until the processing of the fruits under scientific research and formulation of medicine. As we know that dried fruits contain more bioactive chemicals and can be preserved longer before they become unfit, they will be more appropriate in a controlled scientific experiment. This is the reason why there was a deliberate selection of dried fruits instead of fresh achenes. During handling and storage, the sample was observed carefully to maintain its quality until the start of the processing.

Nevertheless, *Withania sonnifera*, also known as ashwagandha was available in its fresh form at Sagar, Madhya Pradesh in the local markets in India. Due to the availability of ashwagandha roots in large amounts that are also readily available, and extensively cultivated in this region, this location was chosen. To provide that only the finest, strongest were select to this. Research, the roots were laboriously cut up. Roots that appeared to be free of contamination, rotting, or physical damage were chosen only because it might lead to loss of bioactive composition of the roots and the accuracy of pharmacological outcomes. The indicated root bark was given special attention. Also, the harvesting was to be on the optimal season of the *Withania sonnifera* when the concentration of its useful chemicals is maximized. In the case of rigorous scientific research, it was a necessity to have such a selection at the season to guarantee optimum potency and reliability. Since in psychopharmacological studies, plant secondary metabolites are prone to decomposition, the prime objective of this study was to develop a safe and reliable bioassay procedure which would ensure high experiment repeatability with fresh and high-quality root materials.

3.1.2 Authentication

The botanical authenticity of the components that were used in this study, *Piper longum* and *Withania sonnifera*, were carefully identified and confirmed. This involved an extensive examination of the morphological features of the plants including their leaves, stems, flowers, and fruits and was compared to the taxonomic keys and the usual botanical descriptions of the plants in the Indian flora books. This procedure has removed the chances of using incorrect or irrelevant plant which may undermine the credibility of the scientific inferences, by making sure that the plants were properly sorted by botanical nomenclature and assigned to the correct species. Due to the fact that the chemical composition, biological properties and pharmacological activity of different species and even within a single species may vary it was necessary to identify them correctly. Misidentification possibility can impair credibility and reproducibility of the research and produce false results. In order to maintain authenticity, voucher specimen of the two plants was prepared and stored in herbarium of Botany, Swami Vivekanand University, Sagar. These specimens were assigned unique catalog numbers (SVNU/MISC/0203 *Piper longum* and SVNU/MISC/0204 *Withania sonnifera*) as reference to be kept and used in future verification and discovery. The preservation of these specimens encourages the

transparency and scientific soundness of the study because they can be reexamined in the case of any doubts or in the event there is a need to verify it in future. The study will make sure to adhere to these authentication standards to be able to guarantee that the findings will be based on real and properly identified samples of plants.

3.2 Preparation of Plant Extract

3.2.1 Selection of Plant Parts

The dried fruits were selected to extract in *Piper longum* extraction due to the fact that it is very familiar with having the highest concentration of active constituents especially the abundant piperine. *Piper longum* is a major bioactive component of *Piper longum* and has been reported to demonstrate various activities of pharmacological activity. The research has pointed out that much of the medicinal properties of plant including antioxidant, anti-inflammatory and anticancer properties can be attributed to piperine. Furthermore, the bioavailability of other molecules, particularly drug absorption, can be enhanced by piperine, and this can be applied in designing various drug formulations. Dried fruits Have a better concentration of piperine than other parts of plant, they are used most to extract these bioactivities. Moreover, the fruits are applied in various traditional medicine systems since time immemorial in respect to various diseases like gastrointestinal disorders, respiratory diseases, or chronic pains. Their interactions with other drug substances continue to add to its significance in the manufacture of herbal drugs. Based on these factors, dried fruits of *Piper longum* would be the most desirable substance to use in the extraction process, to extract and use piperine in an efficient way. In the same manner, in the case of *Withania sonnifera* (Ashwagandha), roots are chosen as the primary part of the plant to extract bearing in mind the historical trends of its use and the scientific proof of its therapeutic effects in recent times. *Withania somniferous* or ashwagandha is the plant of which roots are highly active in Ayurvedic medicine due to its adaptogenic properties to resistance to stress, anxiety, and fatigue. The withanolides, which are rich roots, are steroidal lactones with anti-inflammatory, anticancer and neuroprotective properties among a plethora of other pharmacological actions. Among them, their alleged antidepressant and anxiolytic effects are of specific interest to recent research in the literature. Moreover, alkaloids are also found in roots of the plant and are believed to contribute to the overall therapeutic effect of

Withania somniferous, therefore, its historical application as an herbal remedy in mood disorders, mental impairment and even immunomodulation. The level of those bioactive compounds is proposed to be higher in rhizomes than in the rest of the plant like leaves and stems. Therefore, roots of *Withania sonmifera* are selected to be extracted because they are superior and more effective to extract the medicinal effects of the plant particularly to stress among others.

3.2.2 Extraction Method for *Piper longum*

Dried fruits of *Piper longum* (450 g) were powdered using a mechanical grinder. The powder (100 g) was extracted using cold maceration method with the following protocol:

1. **Maceration:** Plant material was soaked in 95% ethanol (1:4 w/v ratio) at room temperature for 72 hours
2. **Extraction cycles:** Four extraction cycles were performed
3. **Filtration:** Each extract was filtered through Whatman No. 1 filter paper
4. **Concentration:** Combined filtrates were concentrated under reduced pressure at 40°C using rotary evaporator (Buchi Rotavapor R-200, Switzerland)
5. **Yield:** Approximately 45 g of dark brown residue was obtained

Fractionation: The dried extract was successively fractionated using liquid-liquid partitioning with solvents of increasing polarity:

- Hexane
- Dichloromethane (CH₂Cl₂)
- Ethyl acetate (EtOAc)
- n-Butanol (BuOH)

Each fraction was dried under reduced pressure and stored at 4°C until further use.

3.2.3 Extraction Method for *Withania sonmifera*

Fresh roots of *Withania sonmifera* (500 g) were processed as follows:

1. **Cleaning:** Roots were washed thoroughly with tap water followed by distilled water

2. **Drying:** Washed roots were shade-dried for 7 days
3. **Grinding:** Dried roots were pulverized using mechanical grinder
4. **Soxhlet extraction:** Powdered material was extracted with methanol using Soxhlet apparatus for 48 hours at 60-65°C
5. **Filtration:** Extract was filtered through Whatman No. 1 filter paper
6. **Concentration:** Filtrate was concentrated under reduced pressure at 40°C
7. **Fractionation:** The concentrated extract was successively fractionated with:
 - Petroleum ether
 - Chloroform
 - Ethyl acetate

AGG Preparation: The Ayurvedic method of preparing the ghee (clarified butter) Ayurvedic extract methanol and adding it to ghee created Ashwagandha Ghrita (AGG).

3.3 Qualitative Phytochemical Screening

The presence of various phytoconstituents as some standard processes was carried out using ethanol extract of *Piper longum* as well as methanolic extract of *Withania sonnifera* in terms of qualitative phytochemical screening as discussed below:

3.3.1 Test for Alkaloids

Mayer's Test: Only a small amount of Mayer reagent (potassium mercuric iodide solution) was applied on 2 mL of the plant extract. The precipitate was yellow and this meant that it had alkaloids.

Wagner's Test: Wagner reagent (iodine in potassium iodide) was introduced in small proportions of the 2 mL of the plant extract, and the formation of a brown/ reddish-brown color was a sign of alkaloids.

Dragendroff's Test: Few drops of the reagent of potassium bismuth iodide (Dragendroff solution) were added to 2 mL of plant extract. appearance of red precipitate was a sign of alkaloids.

3.3.2 Test for Flavonoids

Shinoda Test (Magnesium Hydrochloride Reduction Test): A few pieces of magnesium ribbon were added to the 2 mL of plant extract and dropwise addition of concentrated hydrochloric acid was carried out. Pink, scarlet, or crimson color was an indication of flavonoids.

Alkaline Reagent Test: A little amount of sodium hydroxide solution was added to 2 mL of plant extract. Formation of an intense yellow color that turned colorless upon addition of few drops of dilute acid indicated presence of flavonoids.

Lead Acetate Test: To 2 mL of plant extract, few drops of 10% lead acetate solution were added. Formation of yellow precipitate indicated presence of flavonoids.

3.3.3 Test for Tannins

Ferric Chloride Test: Few drops of 5% ferric chloride solution were then added to 2 mL of plant extract. When the color was blue-black or green-black, it was evidence of the presence of tannins.

Gelatin Test: A 1% solution of gelatin with sodium chloride (100 ml plant extract) was added. White precipitate was formed which is an indication of tannins present.

3.3.4 Test for Saponins

Foam Test: To 5 mL of plant extract, 5 mL of distilled water was added and shaken vigorously in graduated cylinder for 15 minutes. Development of continuous foam layer (1 cm) was a sign of the presence of the saponins.

Hemolytic Test: Plant extract drop was pipetted onto the microscope slide that was suspended with fresh human red blood cells in saline. The presence of saponins was evidenced by hemolytic of red blood cells that was under the microscope.

3.3.5 Test for Glycosides

Keller-Killiani Test (Test for Deoxy Sugars): To 2 mL of plant extract, 1 mL of glacial acetic acid, one drop of 5% ferric chloride, and concentrated sulfuric acid were added. Appearance of reddish-brown color at junction of two layers & bluish-green color in upper layer indicated presence of glycosides.

Borntrager's Test (Test for Anthraquinone Glycosides): To 3 mL of plant extract, 3 mL of chloroform was added & shaken. Chloroform layer was separated, & 10% ammonia solution was added to it. Pink-red color in the ammonical layer indicated the presence of anthraquinone glycosides.

3.3.6 Test for Terpenoids

Salkowski Test: To 2 mL of plant extract, 2 mL of chloroform & 3 mL of concentrated sulfuric acid were added. Formation of reddish-brown coloration at interface indicated presence of terpenoids.

Liebermann-Burchard Test: To 2 mL of plant extract, 2 mL of acetic anhydride was added, followed by careful addition of 2 mL of concentrated sulfuric acid. Formation of blue-green ring indicated presence of terpenoids.

3.3.7 Documentation of Results

Results of all qualitative phytochemical tests were recorded based on the intensity of color formation or precipitate:

- Strongly positive (+++): Intense color/heavy precipitate
- Moderately positive (++) : Moderate color/precipitate
- Weakly positive (+): Faint color/light precipitate
- Negative (-): No color change/no precipitate

The detailed results are presented in Chapter 4

3.4 Quantitative Phytochemical Analysis

3.4.1 UV-Visible Spectrophotometry

One of the most widely used techniques to study the behavior of absorption of a compound and to identify its electronic transitions is spectrophotometry which is a UV-visible spectrophotometry. The extracts and the isolated compounds of *Piper longum* and *Withania somniferous* were collected by using the Perkin Elmer LS50B spectrophotometer, in order to obtain the maximum absorption (λ_{max}). The λ_{max} is a diagnostic property of the electronic structure of molecules since it is a wavelength

that a molecule absorbs light the most (Lin 2012), which is usually associated with the amount of energy needed to give an electronic transition in the molecule. *Piper longum* was estimated to provide the major active compound, which is piperine that has bioactive properties. The highest absorption level was at 343 nm, where this compound has been recorded to have a peak. The standard solution of Piperine concentration was prepared by using pure piperine (Sigma- Aldrich) of concentrations of 5-50 5g/ml and plotting the standard curve. The standard curve provided enables one to determine the content of piperine present in plant extract with the right degree of accuracy by graphing the absorbance and known concentrations of piperine. This method provided an effective way to measure the concentration of the substance of piperine and it was done using the absorbance of the matter within a wavelength (343 nm) of its 343 nm. Similarly, *Withania somniferous*, which is one more well-known medicinal plant, was the aim to determine the total withanolide content. Withanolides are a family of naturally occurring steroidal lactones present in the plant *Withania somniferous*; chemical whose structures are exploited as its activity as potential chemotherapeutic agents. The maximum wavelength of the withanolides was observed to be at 223 nm that is characteristic of withanolides. To determine the contents of withanolides, withanolide A (purity=95, also obtained at Sigma-Aldrich) was dissolved in the range of 10 100 µg/mL, based on which a standard curve was plotted. Analysis of absorbance at 223 nm was applied to determine content of withanolide in extract by determining standard solutions and plotted absorbance versus 1 divided by 1 as the calibration curve between 215 and 250 nm. UV visible spectrophotometric analysis also proved to be an easy and precise method to determine concentrations of active compounds in the extracts to continue with the further studies and potential application of these plants to pharmaceuticals and therapeutics.

3.4.2 HPLC

The High-Performance Liquid Chromatography (HPLC) was used to analyze bioactive compounds in the extracts of the plant *Piper longum* and *Withania somniferous* in an attempt to quantify the purified bioactive compounds and to determine their purity. The correct and reliable measurements were performed with a Shimadzu LC-20AD system with SPD-M20A diode array detector. In the case of the

Piper longum Linn (long pepper) Phenomenex Luna C18 (5 μ m, 250 $\times$ 4.6 mm) column was used in the separation and characterization of the compounds. The 60% acetonitrile/water (v/v) proportion constituted the mobile phase, which made it possible to separate the bioactive in the extract. The flow rate was kept at 1 mL/min to give the optimum resolution and separation of the constituents. The absorbance wavelength of 343 nm which was the most common bioactive principle found in *Piper longum* was used to perform detection. The quantification of Piperine standard was done by comparison of the retention time and peak area of Piperine standard to sample. The significance of this process is that it gives quantitative value of piperine, which is significant in determining the strength of the plant extract. In the case of *Withania sonnifera* (ashwagandha), a Waters Nova-Pak C18 column (150 $\times$ 3.9 mm 4 μ m) was employed. mobile phase was 0.1% formic acid solution in water and methanol (70:30 v/v). This combination was selected because it is the best in isolating the withanolides, which are the predominant bioactive compounds of *Withania somniferous*. To this end, the flow rate was set to 0.8 mL/min to achieve good flow of the withanolides on the other constituents found in the extract and the withanolides had absorbance maxima at 227 nm hence it could be accurately estimated at this wavelength. The withanolides relative to known standards were measured in terms of retention time and area of the peaks in order to determine the purity and concentration of the compounds correctly. All these analytes are correctly determined and measured in the medicinal plants such that the therapeutic efficacy of the medicinal plant is correctly regulated.

3.4.3 TLC

TLC, Thin-Layer Chromatography is an established and used analytical method of separating and identifying plant constituents which has also been used to separate photochemical. The use of TLC on silica gel 60 F254 plates (Merck, Germany) (as the most common stationary phase in TLC) was done in the work due to its high surface area and the ability to separate a wide range of substances. The plates were developed and separated by the use of appropriate solvent systems as the mobile phase and observed under the UV light at 254 and 366 nm. We find that compounds that naturally absorb in area of the living cell absorb, with the use of UV 254 nm, and that fluorescent spots are indicative of some bioactive compounds which can be easily

identified at 366 nm. TLC solvent system of *Piper longum*, which is a common medicinal plant, was toluene: ethyl acetate (7:3 v/v). This solvent was appropriate in the movement of sample compounds on the silica gel plate. The UV light at 366 nm was focused on and a blue fluorescence spot was found to correspond to the piperine which is the main active ingredient of *Piper longum* because the piperine was shown to exhibit the fluorescence of the UV light at 366 nm. In addition, to establish the existence of the piperine, TLC plate topped with anisaldehyde-sulfuric acid reagent was sprayed which is a general chemical reagent applied to identify the alkaloids and other bioactive components in plant extract. The anisaldehyde-sulfuric acid color of the sample displayed blue-violet color, which is indicative of the presence of piperine and which indicates that the sample is the same. In the case of *Withania sonnifera* (the medicinal herb, also called Ashwagandha), various solvent system chloroform with a mixture of methanol (9:1 v/v) was used. The solvent system was selected to optimize the resolution of withanolides which are the bioactive constituents of *Withania sonnifera*. This was seen as spots of purple color when the TLC plate was sprayed with anisaldehyde-sulfuric acid reagent. Plate was then heated up to 110 °C and allowed to heat up to five minutes to enhance the color formation following spraying. The heat treatment allowed the reactivity between the withanolides and the reagent to take place fully and produce the purple color of the withanolides such that the withanolides were visible on the TLC plate vividly. This was a reliable and sensitive technique of the qualitative establishment of the presence of withanolides in *Withania sonnifera* established the ability of TLC to investigate phytochemicals.

3.4.4 GC-MS

Analysis was done by Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu QP2010 Ultra system, equipped with Rtx-5MS 30 m 0.25 mm, 0.25 0.25 mm) analysis of volatile compounds in the extracts of plants. The system can give correct fractionation and identification of chemical compounds in complex mixtures. Plant extract was injected into the GC system and sample vaporized and brought through column by using helium carrier gas (flow rate: 1.21 mL/min). The GC oven temperature in which a sample was injected was set to program between 60 °C, where the temperature was maintained at 60 °C at a time of 2 minutes to allow equilibration of the sample. Temperature was then programmed to increase at 10 °C /min to final temperature of 280 °C and was allowed to continue a further 10 minutes in order to

have separation of the substances complete. When the temperature increases, volatile components may be forced out of the column depending on their affinities and boiling points of both the stationary and volatile phase of the column and also at the time they volatilize. After leaving the column, the different compounds were then taken to the mass spectrometer and operated in EI mode (ionization energy of 70 eV). Mass spectrometry was used to identify the mass-to-charge ratio (m/z) in the range of 35-500 in order to identify the mass of the compounds. It was determined that the nature of individual chemicals of the plant extracts was by comparing the observed spectra with the mass spectral library of the National Institute of Standards and Technology (NIST). Identification was made by the match of experimental values of the mass spectrum of the degradation products of penicillin with that in the library. The compositions of the plant extracts are fully characterized using this methodology resulting in the identification of interesting bioactive compounds.

3.5 Isolation of Compounds

3.5.1 Preparative TLC Separation

Preparation of individual active plant extracts Preparative Thin-layer Chromatography (PTLC) was used to isolate and purify individual active plant extracts using 60 F254 plates of silica gel (20 x 20 cm, 1 mm thick, Merck, Germany). It began after the silica plates were treated and developed using solvent systems that were known in the literature to utilize the adequate level of separation among compounds. The UV light at 254 nm and 366 nm was used on PTLC plates and this allowed the compounds of various forms to exist in the plates depending on their ability to absorb in this wavelength. The compounds of interest were located and eluted off of the silica gel plate by the bands they were corresponding to. After the samples were scraped the compounds were eluted using an appropriate solvent stripping that enabled full dissolution and extraction of the target compounds. The toluene and ethyl acetate in toluene and ethyl acetate (7:3 v/v) was the mobile phase used in extracting the PTLC piperine using *Piper longum* L (Long pepper). This solvent system was polar enough to permit separation of piperine into a distinct spot with Retention Factor (R_f) value of 0.47. The scrapped band was then eluted by the use of methanol into the piperine which was then further utilized. Mobile phase used was PTLC mixture of chloroform and methanol; 9: 1 (v/v) used to PTLC *Withania*

sonmifera (ashwagandha) extracts. Rf 0.3 -0.6 produced withholds as bioactive compounds of *Withania sonmifera* as bands. These plates were scraped very carefully and transferred to plate, and extracted with chloroform/methanol (1:1, v/v) to extract the withanolides as much as possible. Size-exclusion chromatography on the final eluate was used to further purify or analyze the compound that were eluted, which in this case, were More detailed information = 2mg/ml.

3.5.2 Characterization and Structure Elucidation of Isolated Compounds Using NMR

Nuclear Magnetic Resonance spectroscopy is a potent methodology in investigating structure of organic compounds of interest since it interrogates the manner in which nuclear spins are influenced by magnetic fields. Proton (^1H) & carbon (^{13}C) NMR was done using Bruker DRX-300 at 300 MHz (^1H) and 75 MHz (^{13}C). The ^1H NMR (Hydrogen-1 NMR) spectrum informs us of the hydrogen atoms in the molecule and the ^{13}C NMR spectrum informs us about the carbon atoms. At the frequencies (300 MHz 1Hz and 75 MHz 1Hz) used, good resolution, and sensitivity are obtained, and by monitoring the frequencies all the way through one can observe the signals of various nuclei in the sample. Dissolved samples were in deuterated solvents, i.e. isotopes of hydrogen or carbon which do not give out signals in NMR method. Solvents and mixtures to prepare the samples were deuterated with both CDCl_3 and DMSO-d_6 that are commonly used in NMR spectroscopy as NMR active nuclei which minimize the amount of background noise caused by the solvent. These solvents are also relatively broadly active in solvency to a wide range of materials, which makes them rather handy as well in NMR analysis. The chemical shift was referenced with the use of Tetramethylsilane (TMS) as an inner reference standard to measure the NMR spectrum. TMS is typically chosen due to the high-intensity sharp signals at reference chemical shift position 0 ppm in the ^1H NMR spectrum. This gives a point of stable comparison of chemical shifts between samples and experiments. Overall, this NMR system provides highly fined and precise spectral data of the assignment and structural differentiation through organic compounds.

For *Piper longum*, the isolated piperine was characterized by ^1H NMR and ^{13}C NMR spectroscopy to confirm its identity. The characteristic signals for piperine in ^1H NMR included:

- Aromatic protons: δ 6.98-7.42 ppm (m, 5H)
- Olefin protons: δ 6.44-6.78 ppm (m, 3H)
- Ethylene protons: δ 1.56-3.64 ppm (m, 6H)
- Methoxy protons: δ 3.87 ppm (s, 3H)

For *Withania somniferous*, the isolated withanolides were described using ^1H and ^{13}C NMR Spectroscopy to confirm their identity. The characteristic signals for withanolides in ^1H NMR included:

- Olefin proton at C-2: δ 5.80-5.85 ppm (d, 1H)
- Oxymethine proton at C-22: δ 4.20-4.40 ppm (dd, 1H)
- Methyl protons: δ 0.80-1.40 ppm (s, 15H)

3.5.3 Column Chromatography

Isolation and purification of compounds in the extracts of *Piper longum* and *Withania sonnifera*, isolation and purification of compounds in extracts of *Piper longum* and *Withania sonnifera* was done using isolation and purification by isolating and purifying using Silica gel 60 stationary phase (Fisher scientific, 230-400 mesh). Silica is a strong adsorbent that easily separates the compounds based on their polarity. The wet method was used to prepare slurries of silica gel 60 (70% of loading step) and this step was also done by packing the column with the suitable slurry through wet-packing method by using the suitable solvents that were to be used in the mobile phase (v/v). The solvent is placed in small sections, to create a good, smooth bed of SG. The stationary phase was strongly perturbed by the choice of the solvents used in the mobile phase, which dictated the separation between the compounds of different affinities. The separation of P. longum compounds was performed by gradient elution system in which the initial concentration of the solvent mixture was 1% methanol in dichloromethane, and the proportion of the solvents was changed to 100% methanol in dichloromethane over time. This gradient allowed a general linear variation in polarity and consequently separation of the compounds that had varying solubilities in the mixture solvent. Fractions were pooled and purity of the fraction and composition measured by Thin Layer Chromatography (TLC) during the elution.

TLC is a simple and fast technique of analysis, it assists in visualizing various constituents in each fraction helping in the separation of fractions with similar profiles. Following chromatography, fractions were pooled based on the TLC profiles of the pools and seven fractions (C1 to C7) were retrieved. Among them, fraction C2 that demonstrated the strongest MAO-inhibitory properties allowed achieving the impressive inhibition rate of 99.8% at the concentration of 100mg/mL. This was then further purified by recrystallization with a mixture of dichloromethane and methanol, through which highly pure compounds can be obtained by dissolving the sample in a hot solvent and slowing cooling the solution which prefers the crystallization of the compound. This was then supplemented with piperine (450mg) which is a well-reported bioactive component of *Piper longum* which can be credited to the dermatology and health benefits of stone long pepper. During the procedure in *Withania sonnifera*, column chromatography was performed using various gradient elution system with the methanolic extract. Crude extracts were dissolved in sequential solvent mixtures of ethyl acetate in petroleum ether (10-100% of ethyl acetate) lastly in methanol. The gradient was designed so that it would be able to resolve withanolides (steroidal lactones with known biological activity) as effectively as possible in contrast to non-polar compounds and less-polar compounds present in the extract. Fractions were first checked by TLC before proceeding with the gradient, and withanolide containing fractions were then pooled. The withanolide-enriched fractions were put under preparative TLC, which is an advanced method of TLC that is used when a large quantity of pure compounds has to be isolated. Preparative TLC has also proved useful in the separation of compounds in preparative scale and purification of pure withanolides is necessary to their medicinal values of *Withania sonnifera*. Gradient elution systems and TLC are both efficient in the separation and purification process and enable the isolation of bioactive constituents in a certain and pure state.

3.5.4 Preparing Samples for UV, TLC, HPLC, NMR

The choice of the solvent, concentration and sample treatment (way of preparing the sample) are significant aspects and should be taken into consideration before any of these forms of analysis is applied, so that, to achieve quantitative and repeatable results. Preparation of the sample in UV- visible spectrophotometry involves dissolution of the extract or fraction of the sample in a proper solvent- methanol or

ethanol. The choice of the solvent relies on the solubility of the compound in which we want the compound to be in its entirety in order to record accurate absorbance. Concentration of 0.1 mg/mL is commonly the 0.1 mg/mL concentration of most compounds since it is within the sweet spot of maximized absorbance of the sample neither being too concentrated to over saturate the detector or it being too dilute which would result in too low of absorbance. Sample solution is carefully prepared and therefore contamination or decomposition of the compound is unlikely and in most instances a new solution is prepared each time an individual measurement is taken in order to eliminate inaccuracy. For TLC, there is a difference in the sample preparation. The extract or pure compound is dissolved in methanol or chloroform (50-100%), and the concentration was 10 mg/mL. This is stronger concentration than the general use of it since the sample on the TLC plate is spotted and, as such, the sample on the plate should be extremely small amount of compound that can be observed as it moves the length of the stationary phase. In TLC, the chosen solvent relies on the solubility of the compound in question so that the latter will move onto the TLC well. The chloroform is more appropriate in polar distinctions and the use of methanol is more common with non-polar distinctions. That sample is carefully spotted on this plate using either a hairfine capillary tube or micro-pipette, and much caution is needed, to ensure that the sample is properly applied and is not touching any other spot on the plate; because by this procedure, it is impossible to examine only one or more spots on a plate. In the case of High- Performance Liquid Chromatography (HPLC) analysis, the preparation of the sample is conducted through the dissolution of the extract or isolated compound in HPLC-grade methanol to achieve the concentration of 1 mg/mL Methanol of HPLC grade has to be used so that the separation and detection processes are not interfered by the potential impurities or additives. The concentration of the sample 1 mg/mL is chosen so that the analyte could be in the linear response range of the HPLC detector and could be accurately quantified. Concentrated solution filtered with 0.45 μ m membrane filter before injection to eliminate particles or impurities which may plug HPLC column or mess up analysis. Filtering plays a very important role to sustain HPLC systems life and to avoid clogging and assist in generation of reliable results, day after day.

In NMR (nuclear magnetic resonance) spectroscopy (EPS, NOESY, HSQC, HMBC), compound (usually, 5-10 mg) is placed in NMR tubes and dissolved in 0.6 mL of

deuterated solvent (CDCl₃, D₂O or DMSO-d₆). Deuterated solvent is used on, the solute since it does not undergo proton NMR interference. In 5 mm NMR tube (common size on the majority of NMR machines), in which the solution is placed, the solution volume is large enough to be detected, but not so large as to cause issues with signal quality. Dissolution of sample in deuterated solvent is essential to form sharp and neat spectra, which is crucial in the proper structural investigation of the compound. None of these methods of preparation is meant to improve the analysis of the sample with the most promising technique to provide the best possible sample analysis (and thus the most reliable and accurate data) with the technique in question, nor to provide the best possible data on the sample, data collected on the sample is data on the sample, and is the best possible data on the sample.

3.5.5 Acute Toxicity Study

Acute toxicity test was conducted based on OECD (Organization for Economic Co-operation and Development) 423 guideline, which is known as Acute Toxic Class Approach. The method is generally employed in the ranking of the relative harmfulness of compounds, provides a systematized and standardized procedure of assaying the toxicity capability of the test material. Experimental animals used in the current study were female Wistar rats as they are the species of same strain that is commonly used in conducting toxicological studies. The normal body weight of adult rats that are used to study rats of this nature is 200 g. There a total of six animals to allow data analysis. The test substances were administered to rats who were starved 12-16 h prior to the experiment. This starving condition assures of a free gastrointestinal tract and enhanced absorption of the test article. The animals were also starved between 3 to 4 h after dosing in order to exclude the chances of food intake having an effect on absorption and metabolism of the test compounds. Rats were allowed to drink water freely during fasting to ensure that they did not experience dehydration. The sample materials used in the current study were extracts of two medicinal plants *Piper longum* (long pepper) and *Withania somniferous* (ashwagandha). Plants have versatile pharmacological applications and acute Toxicity Research was conducted to establish their extent of safety on long-term oral intake at excessive dosages. The dose used in the acute toxicity test was 2000 mg/kg body weight of each extract, which is regarded to be high dosing during the toxicological screening. The extracts were administered orally, which is common

practice in finding the toxicity of orally administered substances. The animals were periodically monitored with regard to toxicity after the administration of test compounds. This was monitored by observing the animals individually in case of any behavioral aberrations like depression, abnormal behavior and overt suffering. Deaths were also enumerated and any animal that was sensitively responsive to injection recorded. Careful observation was done on the animals.

The animals were then closely monitored at various intervals over the ensuing 24 h, with the initial 4 h being the most critical as this is at the time when most of the acute responses to allergens are evident. This is because such a long time of observation gives a chance to see any sub-acute or chronic effects that might arise after the acute toxicity. The health status of animals was observed during the process and those that exhibited over-suffering or showed dire side-effects were put to rest and this was done in a way that conformed to ethical standards. This study provided useful information about the safety and potential toxicity of standardized extract of *Piper longum* and *Withania sonnifera* as well as provided a more profound insight into their risk profile in relation to acute toxicity. The results of such research are significant to endorse the safe usage of these natural compounds, particularly in medicinal and curative use.

3.6 Preparation of Test Drug

3.6.1 Selection of Test Dose

Based on results of acute toxicity study and literature data, following doses were selected for study:

For *Piper longum*:

- Low dose: 10 mg/kg body weight
- High dose: 200 mg/kg

body weight For *Withania*

somniferous (AGG):

- Low dose (T1-AGG): 20 mg/kg body weight
- High dose (T2-AGG): 40 mg/kg

body weight For combination therapy (T3-

AGG + Imipramine):

- 10 mg/kg body weight each

3.6.2 Preparation of Standard Dose of Drug

Imipramine hydrochloride (Sigma-Aldrich) was used as standard antidepressant drug at dose of 15 mg/kg body weight. Drug was dissolved in normal saline (0.9% NaCl) and administered intraperitoneally (i.p.) 60 minutes before the behavioral tests.

3.7 Disease Induction and Design of Experiment

3.7.1 Experimental Animals (24 Wistar Rats)

The albino wistar rats of weight (180-220g) were selected to study. They were the animals acquired in animal house of Guru Khalsa Institute of Science and Technology Jabalpur (M.P.). The animals were housed in standard polypropylene cages of solid bottom (conventional when it comes to rodent studies due to their durability and easiness in cleaning). These cages were kept under a well-being condition of test animals throughout the test process. Room temperature was maintained at $25 \pm 2^\circ \text{C}$, which is the best temperature of laboratory animals. The humidity in the room was also kept at 45-55% – the ideal level of keeping the rodents in the room because they will not become dehydrated and their skin will be in good condition. Lighting was also provided evenly, where 12/12 hour day/night cycles were used to simulate natural variations in day and night, as these are required to maintain the body clock of the animals hence maintaining their health. The diets of the rats were of a balanced mixture of the feed ingredients with all the essential nutrients necessary to grow and maintain them (standard laboratory diet). They even had ad libitum access to food and water (they could eat and drink any time they wished). Animal research is known to be full of food and water that is never withdrawn.

The rations provided were meant to meet the nutritional requirements of rats so that the rats grow and develop as well as their health persists with the duration of the current research. Acclimation Rats that had at least one week acclimation to the laboratory environment were used in the experiment. Acclimatization is also relevant

in animal work since it reduces the stress and anxiety levels that are related to inducing the animals into a new environment. Through this acclimatization, the animals got used to laboratory environment, conditions under which they were housed and the procedure used to handle them. This time must be given to provide assurance that there are no contaminates of the experiment due to stress or the environment of the experiment (which can be the case because of sudden change in environment of rats). This approval is critical in ensuring that the research work was carried out under ethical guidelines and on guidelines governing care and use of animals to perform experiments. Moreover, the experiments were conducted based on the principles of care and use of laboratory animals and the protocol was agreed upon by ethic committees on animal experiments of the research institution that were issued based on the goal of ensuring that the animals were kept in such a way that they were not distressed, suffering and in pain. These processes are in line with the global standards of ethics and animal welfare during the experiment. The animals were randomly divided into six groups (n = 6 per group):

- Group I: Control (Normal saline, 0.01 mL/g)
- Group II: Standard (Imipramine, 15 mg/kg, i.p.)
- Group III: T1-AGG (*Withania sonnifera*, 20 mg/kg)
- Group IV: T2-AGG (*Withania sonnifera*, 40 mg/kg)
- Group V: T3-AGG + Imipramine (10 mg/kg each)
- Group VI: *Piper longum* (200 mg/kg)

3.8 Experimental Animal Model

3.8.1 In Vitro Models Neurotransmitter Release Assays

Procedure of neurotransmitter release Synaptosomes were made out of the male rat (ICR) cerebral cortex. One, the animals were slaughtered in a humane manner that followed dislocation of the neck as a normal practice to reduce pain and humaneness. The brain was euthanized, and the cortical tissue was quickly removed out of the brain. This was subsequently homogenized in 0.32 M sucrose that maintains osmotic

balance during the homogenization as well as suppressing cell lysis. Cellular components were once more suspended and homogenated at 1000 g to 10 min at 4 °C to separate the tissue fragments and large cellular debris of the supernatant with smaller cellular particles. This centrifugation step is high speed and is used to enrich the crude synaptosomal pellet which consists of the synaptic vesicles, mitochondria and other membranous structures that are related to the release of neurotransmitters. The pellet of synaptosomes was gently homogenized in Krebs-Ringer solution (CR) commonly used to sustain cells in vitro. The buffer contained important salts and glucose that gave a physiologically realistic setting with pH of 7.4 similar to the pH of the brain extracellular fluid. The neurotransmitter ([³H]-5-HT, [³H]-NE or [³H]-DA) was preloaded into the synaptosomes in the attributed the neurotransmitter release experiment, respectively, an excess of radiolabel was employed. The release of the neurotransmitters in the experiment was monitored by using these labeled compounds. Incubation was done at 37 °C in 15 min to give the synaptic vesicles time to fill the neurotransmitters. Following this wash, synaptosomes were rinsed in order to eliminate free and non-internalized radio-labeled neurotransmitter. Synaptosomes were washed and subsequently preincubated using various doses of plant extracts or pure compounds in a 15-min incubation. This pre-incubation was done to investigate the potential activity of these compounds in the discharge of neurotransmitters. Synaptosomes stimulation was conducted by adding 15 mM KCl (enough to depolarize the synaptic membrane and trigger the release of the neurotransmitters already loaded to the synaptic vesicles to the extracellular medium). This was stimulated over a period of 5 minutes, and this was a conventional action potential that would release neurotransmitters in vivo. Due to the high rate of filtration of the synaptosomes through glass fiber filters, the reaction was interrupted. This process washed the synaptosomes out of the solution and the released one only.

Radioactivity that corresponded to filters was taken as the amount of neurotransmitter released in the procedure. Measurement of [³H] co-transmitters is then done and the decays conditioned by a scintillation counter of the radiation emitted by [³H]-labeled transmitters used to measure the extent of neurotransmitter released by the stimulation as well as the way it has been altered by plant extracts or compounds. Such assay proved helpful to comprehend neurotransmitter release, and how pharmacological agents might affect the transmission through the synapse, the results of this

experiment could be used to corroborate the effect of the different compounds on the neurotransmission, and its applicability to petrochemical signaling in the brain.

Enzyme Inhibition Assay (MAO Inhibition)

The Crude mitochondrial fraction of the mouse brain prepared according to Naoi et al with slight modification was used to prepare Mao (Monoamine Oxidase) inhibition assay. Male rats were cervically dislocated and brains were dissected quickly on ice to minimize the effects of enzymes on degradation obtained brain tissues were homogenized using 0.25 M sucrose (10 volumes) to mix up cell structure in the brain and liberate intracellular content, including mitochondria. the homogenate was centrifuged at 1000 g at 4 C during 10 min to remove larger cell debris and nuclei but leave supernatant containing smaller organelles, including mitochondria. Higher speed (16,000 g, 30 minutes at 4 C) centrifugation of this supernatant was also done to achieve the concentration of mitochondrial pellet that is enriched with MAO enzymes. After centrifugation, the mitochondrial pellet was re-suspended in the solution of sucrose to preserve its structure and was kept in -80 C till the assays to preserve the activity of the stored enzymes. Fluorescence method was used to define the activity of the MAO using kynuramine as substrate. Each experimental point was incubated at 37 ° C with reaction mixture containing 50 0L of mitochondrial fraction (1 mg/mL protein), 50 0L of kynuramine (0.1 mM) and 50 0L of various concentrations of plant bioproducts or compounds under study dissolved in 100 mL potassium phosphate buffer (pH 7.4) to a final volume of 200 0L. 30 minutes to enable action of MAO enzyme to work on kynuramine substrate. To prevent any further enzymatic activity, 100 3L 2N NaOH was used to quench the reactions and the fluorescence intensity of 4-hydroxyquinoline product was measured in 320 nm excitation wavelength and 380 nm emission wavelength (Perkin Elmer LS50B fluorescence spectrophotometer). The latter enabled it to determine the activity of MAO through the fluorescence of the product.

Selectivity of the MAO inhibition was also tested with positive controls (1 mM), so both selective nonspecific MAO inhibitors (l-deprenyl (selective to MAO-B), and clorgyline (selective to MAO-A). These inhibitors were used to determine assay specificity to MAO is forms and ensured that the effects were due to inhibition of MAO, as opposed to other sources of enzymatic acts. Additionally, a dialysis

experiment was also done to examine the potential binding of plant extracts/ isolating compounds with mitochondrial MAO enzyme, in experiments, mitochondria were incubated with known compound piperine at 37 °C over 30 minutes. Mitochondrial suspension was then incubated followed by 4 h dialysis at 4 °C using 100 mM potassium phosphate buffer at pH 7.4 to equilibrate compounds and diffuse across dialysis membrane. The same fluorometric technique above was used to measure the MAO activity after dialysis to ensure that the change in the inhibition of the enzyme was actually the change of the enzyme due to the dialysis. It was a type of experiment that allowed a wholesome characterization of the MAO inhibitory potential of plant extracts or individual compounds and this allowed a clearer understanding of their pharmacological capabilities of treating neurodegenerative diseases and other pathologies with an imbalanced monoamines metabolism.

3.8.2 Vivo Models Behavior Models Forced Swim Test (FST)

The Forced Swim Test (FST) is a well-known behavioral test that was invented to study the extent of despair/helplessness of rodents and is primarily used to prescribe the potential (anti)depressant effect of drugs. It relies on the experiment conducted by Porsolt et al., wherein the rats are made to swim freely in water (without restraints) in glass cylinder with water (so that the rats cannot escape) in the cylinder is 20 cm across and 50 cm in height, and the level of the water is maintained at constant temperature (25 to 27 °C) to ensure it is at its optimum level so that the rats do not experience stress due to water temperature. The investigator in the meantime observes and scores the behavior of the rat such as immobility time. Immobility is characterized by a condition of no swimming/escape movements with exception of slight movements to avoid drowning. This immobility is interpreted as a behavioral hopelessness or uninspired has been widely used as an indicator of depression-like.

In acute test, the test substances are administered only once, 60 min prior to test to enable the substances to be absorbed and to exert its action to the animal. FST is then administered as follows; the immobility duration of the rat is timed of the last 4 min of the swim, since it has been argued that the behaviour in the last 4 min is more predictive of the emotional state of the animal, and that after 4-min, the animal becomes less active and presumably more willing to exhibit signs of despair. acute model is also useful in assessing the immediate and short-term action of the test

substances and allows the comparison of their behaviour to that of the control or reference group, often, including conventional antidepressants. Alternatively, the chronic study is more prolonged therapy, in which the test animals are gavaged with the compounds of interest on a daily basis over a fourteen days period. This is a procedure that is developed to examine repeated drug exposure and how these substances can bring about a long lasting change in behavior. The FST is conducted 60 min after administration of the last dose on the 14th day of administration in the acute study as the acute study is of cross-sectional nature, and therefore, provides an overview of the potential effect the chronic exposure of the test agents may have on the behavior of the rats. Chronic FST is chronic exposure of depressive like state chronically and especially the effects of chronic exposure of drug like antidepressant, in which not only the efficacy of the drug may be tested but also the potential side effects such as those of the antidepressant drugs of chronic treatment. Time of immobility is of crucial significance to both acute and chronic tests analysis as one of the key factors of the animal's behavioral performance. The shortening of the period of immobility following treatment in most of these studies is taken as an antidepressant-type response and the implication is that the test compound has overcome the despair-like behavior. Conversely, increase in immobility is an indicator of worsening in behavior indicative of depression. Thus, FST has been employed as a screening system in the activity of a substance with potential antidepressant activity and a model to study the mechanism of mood disorders.

Tail Suspension Test (TST)

TST is a well-known test (Graph Pad software, Inc., USA) Graph Pad Prism 8.0 rodents, primarily rats, and requires the measurement of the immobility response in rats when suspended by their tail. This approach was initially outlined by Steru et al. and involves hanging the rats by their tails by adhesive tapes put on a horizontal bar that is placed approximately 50 cm above the ground. It is achieved by placing people in a controlled, sound-proof setting to minimize the possibility of external distractions and stressor to affect behavior and ensuring that the behavior in question when it is recorded is actually a response to the test and not of the environment in general. The chamber used is a box of 25 × 25 × 30 cm which provides.

The procedure has the duration of 6 min with rats suspended. The test primarily focuses on gauging the immobility which is defined as a lack of any voluntary movement of the limbs and body, other than breathing. Immobility is used as the behavioral despair measure, which is one of the main aspects of the depression-like conditions in rodents. The TST is used in both chronic and acute experimental techniques to examine the effect of different drugs in depressive-like behavior. Under the acute study system, the test subjects, rats, receive one dose of the compound, 1 hour before the test. The choice of this window is partly due to allow the drug to take effect but still not to affect the behavior in the rats too late or too early to manipulate the behavior. The acute study is suitable to observe the acute effects of a material on behavior which are short term, and is generally utilized to provide a screen of the effectiveness of a prospective antidepressant agent. Alternatively, the chronic study is the exposure to the test material on 14 consecutive days. This protracted dosing schedule serves as a template of chronic drug exposure, which allows investigation of the extent to which the substance might induce long-term behavior change. The TST test is performed on the rats 60 min following the final dose of the 14 days treatment period. This design is particularly of importance where testing of substances with an expected combination or delayed effect is to be done, in order to provide a long-term view of the treatment impact on the behavioral reactions of the animals. Time immobility during the final 4 min of test is measured during the acute and chronic experiments. It is believed that this period is the most valid indicator of a depression-like behavior, because it is an expression of the tendency of an animal to adopt a withdrawn and motionless position in the face of stress or discomfort. Immobility is also regarded as an absence of coping response and a helpless state, which is similar to features of depression in humans. The immobility measure using varying experimental conditions can be used to determine the effect of various substances (i.e., putative anti-depressant drugs) on depression-like behavior. The Tail Suspension Test is a sensitive index of the effects of pharmacological interventions on the control of mood and behavior, and is therefore a useful test in preclinical studies of potential therapeutics in mood disorders.

Open Field Test

The spontaneous motor activity of animals was measured using Open Field Test

(OFT) that helps to explore and general locomotor activity of an animal. The arena used in the experiment was a circular arena (80 cm) with three concentric circles subdividing it into 36 equal segments, thus creating a grid on the floor, which made it easier to observe the movement of animals in the arena systematically. Animals were isolated and in middle of the arena and permitted to roam around the arena in 5-min. length of time in which, the number of squares moved between one and another were counted as an index of locomotion of the animals in this period. This was an activity that was evaluated to determine how the animal behaved spontaneously and how the tested substances affected its behavior. Test was conducted 60 minutes after administration of test substances and enough time was provided to ensure that the substances had an effect to the behavior of the animal. Timing was also a factor of great significance as this distinguished between general elevation of locomotion as a result of test compounds and more specific antidepressant effects. In case the results of this test were compared with other tests of behaviour (e.g., Forced Swim Test (FST) and Tail Suspension Test (TST), researchers could determine the extent to which the effects were caused by the increase in the physical activity (e.g. by the normalization of Res) or indicated the presence of the specific changes in the mood and behaviour typically caused by antidepressant effect. This was an experimental study meant to illuminate how these substances would impact on the physical and emotional conditions of the behaviors of the animals.

3.9 Biochemical Analysis

Biochemical analyses were used to determine the effects of plant extracts and isolated compounds on concentrations of neurotransmitters in the brain. After the behavioral testing, the animals were cervically dislocated and their brains were removed as soon as possible on ice and sliced to remove and isolate striatum, hippocampal and cerebral cortex. Measurement of neurotransmitters followed the collection of supernatants. 5-HT was measured by Haley and McCormick (1957) method. 34617 was centrifuged at 14,000 g/min at 4 ° C after homogenization in 0.1 M perchloric acid and 0.1 mM EDTA. The level of 5-HT, NE, and DA could be detected with the help of Graph Pad Prism 8.0 (Graph Pad Software, Inc., USA) in combination with high-performance liquid chromatography.

3.10 Plan of Work

This is the current research, which has been conducted to conduct an entire comparative research on the antidepressant effect of *Piper longum* (fruit) and *Withania sonnifera* (root), with different pharmacological parameters. The overall research methodology is structured into a series of associated steps:

Research Envisaged

This study will seek to undertake a systematic comparative research of *Piper longum* (Long pepper) versus *Withania sonnifera* (Ashwagandha) in terms of their possible antidepressant effect. Traditionally Ayurvedic medicine has long been treated by centuries to treat several other conditions using both plants, but the precise mechanism of action, the relative efficacy, and the possible therapeutic value in depression are not fully understood. The study will focus on a compound methodology of a combination of phytochemical, in vitro biochemical, in vivo behavior and mechanistic studies to fully compare and contrast their antidepressant capabilities. The research will give the crucial details of the bioactive constituents that make antidepressant effects, understand their specific mechanisms of action, establish their best doses, investigate their safety profiles, and determine whether they can be used as the sole treatment or supplemental to the conventional antidepressants. The comparative element of the work will emphasize the special properties and possible clinical use of all plants in treatment of depression.

Collection and Authentication of Plant Material

This study is based on acquiring and validating quality vegetal materials so as to have the correctness and reliability of all subsequent analysis.

***Piper longum* (Long pepper):** *Piper longum* (Long pepper): *Piper longum* is a spice that will be sourced carefully in the form of dried fruits in a recognized herbal pharmacy located in Sagar, Madhya Pradesh India, which will meet the standards of quality control and well documented origin of the plant. Dried fruits are not only preferred to fresh fruits, but they contain more bioactive compounds and are also more stable. To ensure that the integrity of the bioactive constituents is maintained and all the chemical elements will not be degraded before extraction, the samples will be stored in dark condition at 4 °C

***Withania sonnifera* (Ashwagandha):** Local markets in Sagar, Madhya Pradesh, India which is known to produce high quality Aswagandha will be used to collect fresh roots. The selection will target healthy and free of disease specimen and at the most desirable harvest season when bioactive concentration is at a peak. The root bark will be considered with a special attention as it contains large amounts of active constituents.

Authentication Process: Both vegetal materials are going to go through stringent botanical verification by:

- Intensive morphological analysis of the physical traits vis-a-vis the taxonomic literature.
- Microscopic analysis of the cell structures.
- Comparison using authenticated herbarium specimen.
- Allocation of repeated voucher specimen numbers (SVNU/MISC/0203 of *Piper longum* and SVNU/MISC/0204 of *Withania sonnifera*)

Preservation: The herbarium to be maintained in the herbarium should be that of the Department of Botany, Swami Vivekanand University Sagar, M.P., which would be used in the future and checked. Such a careful authentication procedure will avoid wrong identification that may undermine the validity of the research results since chemical composition and therapeutic activities may differ across or even within different species.

Preparation of Plant Extracts

The extraction process is critical for isolating the bioactive constituents while preserving their pharmacological properties. Different extraction methods will be employed for each plant based on their specific phytochemical characteristics and traditional preparation methods.

***Piper longum* Extract Preparation:**

1. Mechanical grinding of 450g dried fruits to reduce particle size and increase surface area for efficient extraction

2. Cold extraction with 95% ethanol at room temperature using a 1:4 (w/v) ratio
3. Maceration for 72 hours with four repetition cycles to ensure complete extraction of bioactive compounds
4. Filtration through Whatman No. 1 filter paper to remove solid residues
5. Concentration of combined filtrates under reduced pressure at 40°C to prevent thermal degradation of heat-sensitive compounds
6. Fractionation of the concentrated extract (approximately 45g) through liquid-liquid partitioning with solvents of increasing polarity:
 - Hexane fraction (for non-polar compounds)
 - Dichloromethane fraction (for moderately polar compounds)
 - Ethyl acetate fraction (for more polar compounds)
 - Butanol fraction (for highly polar compounds)
7. Collection and storage of individual fractions for subsequent analysis and bioactivity testing

***Withania sonnifera* Extract Preparation:**

1. Thorough washing of fresh roots with tap water followed by distilled water to remove soil particles and contaminants
2. Shade-drying for 7 days to preserve heat-sensitive bioactive compounds
3. Pulverization using a mechanical grinder to achieve uniform particle size
4. Extraction of 500g powdered material with methanol using Soxhlet apparatus for 48 hours at 60-65°C
5. Filtration through Whatman No. 1 filter paper
6. Concentration under reduced pressure at 40°C
7. Successive fractionation with petroleum ether, chloroform, and ethyl acetate to separate compounds based on polarity

8. Preparation of Ashwagandha Ghrita (AGG) formulation by incorporating the methanolic extract into clarified butter (ghee) after the conventional Ayurvedic practices to increase the bioavailability of active constituents.

The extraction and fractionation steps will be optimally optimized and standardized to guarantee conformity in the composition of the extracts in every extraction and fractionation stage, which is essential in ensuring consistent pharmacological analysis.

Phytochemical Analysis

The detailed phytochemical research will be performed to determine, describe, and quantify the bioactive components of the two plant extracts as the key to the research of the pharmacological actions.

Qualitative Phytochemical Screening: Qualitative phytochemical screening uses a series of general chemical methods to identify the presence of the major phytochemical classes in extracts of plants. The identification of alkaloids is done using tests such as Mayer, Wagner, and Dragendorff. The test by Mayer contains alkaloids because when this test is reacted with potassium mercuric iodide, a yellow precipitate is obtained. The test of Dragendorff gives a red precipitate with potassium bismuth iodide, and the presence of alkaloid is also indicated by the alkaloid test in which iodine is added to potassium iodide giving a brown or reddish-brown precipitate. Identifying flavonoids is performed by the Shinoda test; the test is characterized by the appearance of pink, scarlet, or crimson color when magnesium ribbon and strong hydrochloric acid are used. The lead acetate test and alkaline reagent test also confirm the presence of flavonoid because it yields a yellow precipitate with 10% lead acetate solution and the solution acquires a bright yellow color, and upon the addition of dilute acid, the solution turns colorless. Two techniques that are used to determine tannins are the ferric chloride test and the gelatin test. The ferric chloride test involves the use of 5% ferric chloride to give a blue-black or green-black color, and the gelatin test, which involves the use of 1% gelatin solution that contains sodium chloride that forms a white precipitate. Saponins are identified through hemolytic test, which confirms the presence of saponins by visualizing the hemolysis of red blood cells under the microscope and the foam test, which forms persistent foam when intensely shaken. Glycosides are identified by the

Keller-Killiani test, giving a reddish-brown color where two layers are crossed, one being bluish-green, and the Borntrager test, in which the ammoniacal layer acquires a pink-red color. These are the anthraquinone glycosides tests. Finally, the presence of terpenoids is confirmed using Salkowski test, in which strong sulfuric acid and chloroform are used to form a reddish-brown color at the interface and Liebermann-Burchard test, where the formation of a blue-green ring is observed.

Quantitative Phytochemical Analysis: Quantitative phytochemical analysis involves measuring bioactive compounds with high precision in reliable methods of analysis. It is most commonly used with UV-Visible spectrophotometry where *Piper longum* is studied in terms of its piperine content at a maximum absorption wavelength (maximum absorption wavelength or λ_{max}) of 343 nm. Pure piperine is used to prepare a standard calibration curve in concentration of 5-50 $\mu\text{g/ml}$. In the same way, *Withania sonnifera* is tested on the content of withanolides at 223 nm, and standard curve is built on withanolide A in the range of 10 to 100 $\mu\text{g/ml}$. The phytochemical content of both cases is determined using the values of absorbance as well as the calibration curves of the results. The technique is more specific when using the High-Performance Liquid Chromatography (HPLC)). In the case of *Piper longum*, the separation is done on a Phenomenex Luna C18 column (5 μm , 250 $\times$ 4.6 mm) through mobile phase of 60 per cent acetonitrile and water at a flow rate of 1 mL/min, which was detected at 343 nm. In the case of *Withania sonnifera*, the separation is performed on Waters Nova-Pak C18 column (150 3.9 mm 4 μm) using the mobile phase of 0.1% formic acid in water and methanol (70.30 v/v), and the flow rate is 0.8 mL/min with a 227 nm detector. The quantification relies on the comparison of the retention times and peak areas with the authentic standards. Identification of the components is also done using Thin-layer Chromatography (TLC) on silica gel 60 F254 plates. *Piper longum* is cultivated in a solvent mixture of toluene and ethyl acetate (7:3 v/v), and observed under UV light at 254 and 366nm and improved by spraying with anisaldehyde-sulfuric acid reagent.

The samples of *Withania sonnifera* are grown in chloroform and methanol (9:1 v/v), then sprayed with anisaldehyde-sulfuric acid and incubated at 110°C at 5 minutes. The values of retention factor (R_f) are identified with the aim to distinguish particular components. As well, Gas Chromatography- Mass spectrometry (GC-MS) provides a detailed profiling, and the device used is a Shimadzu QP2010 Ultra system, and the

column used is Rtx-5MS column. The temperature will be programmed between 60 ° C (holding 2 minutes) to 280 ° C at a rate of 10 ° C /min with a holding time of 10 minutes at the high temperature. The carrier gas is Helium and flow rate is 1.21 mL/min. Mass spectrometry is done using electron ionization (EI) mode of operation with 70 eV mass-to-charge ratio (m/z) range. Identification of compounds is done by comparison of their mass spectra with the database of NIST library, which also makes sure that the phytochemical constituents are properly characterized. Such thorough phytochemical examinations will give in-depth profiles of the bioactive compounds in the two plants, which will form the basis of knowing their pharmacological actions and standardisation of extracts that would be used in future researches.

Isolation and Characterization of Bioactive Compounds

The isolation and structural elucidation of key bioactive compounds will be crucial for understanding their specific contributions to the antidepressant effects of the plant extracts.

Preparative TLC Separation:

1. Use of silica gel 60 F254 plates (20 x 20 cm, 1 mm thick)
2. For *Piper longum*: Development with toluene:ethyl acetate (7:3 v/v), identification of piperine at R_f value of 0.47
3. For *Withania somnifera*: Development with chloroform:methanol (9:1 v/v), identification of withanolides at R_f values 0.3-0.6
4. Visualization under UV light (254 and 366 nm)
5. Scraping and elution of targeted bands with appropriate solvents
6. Concentration and purification of eluted compounds

Column Chromatography: Column chromatography can be recognized as an important method of isolating and purifying plant bioactive compounds. When doing this, columns are being made in silica gel 60 with a mesh size of 230-400 and serves as the stationary phase. To separate the compounds of *Piper longum*, gradient elution technique is used with the initial percentage of methanol in dichloromethane as 1, followed by a steep increment on methanol to 100 percent. In *Withania sonmifera*,

the extraction is performed with ethyl acetate in petroleum ether and then the percent is increased to a hundred and then it is extracted by the use of methanol. The collections are systemically collected and observed with Thin-Layer Chromatography (TLC) in order to profile target compound presence. Fractions in the TLC that are detected bioactivity are then submitted to another purification process, such as recrystallization, to increase purity. The process will allow isolating large amounts of major phytochemicals including about 450 mg of piperine in *Piper longum* and different withanolides in *Withania sonnifera*, making them applicable in further analytical and pharmacological research.

Structural Characterization:

- **Nuclear Magnetic Resonance (NMR) Spectroscopy:** The nuclear Magnetic Resonance (NMR) spectroscopy is considered an effective method of structural elucidation with the help of Bruker DRX-300 spectrometer. During this, proton (^1H) NMR is carried out when 300 MHz is used to analyze the hydrogen atoms in the molecules with carbon (^{13}C) NMR being done at 75 MHz to identify the carbon structure. The samples are made in deuterated solvents including CDCl_3 and $\text{DMSO}-d_6$, and tetramethylsilane (TMS) as the internal reference of the chemical shift. In the case of piperine, the identified characteristic signals are aromatic protons at the range of 6.98-7.42 ppm and olefinic protons at the range of 6.44- less than. 6.78 ppm ethylene protons, 1.56-3.64 ppm, and 3.87 ppm, methoxy protons. In the case of withanolides, the following signals are observed, an olefinic proton in C-2 with 9.8 kHz at 80 -5.85 ppm, an oxymethine proton in C-22 with 9.2 kHz at 40 -4.40 ppm, and methyl protons at the range of 0.80-1.40 ppm. These typical NMR spectra give the conclusive analysis of the structural identity of the isolated compounds.
- **Mass Spectrometry:** The mass spectrometry is applied to get the mass weight and fragmentation pattern of the compounds that are purified giving an additional confirmation of the elucidation of these structures. In the case of piperine, mass spectrometry analysis is proving that the molecular formula is $\text{C}_{17}\text{H}_{19}\text{NO}_3$ with a strong molecular ion at m/z 285. The disintegration pattern also supports the existence of major structural elements that are

peculiar to piperine. Likewise, the withanolides are examined to confirm the molecular formulas and structural information of the molecules using characteristic fragmentation pathways that confirm their membership of the steroidal lactone family. This molecular-based assurance is essential in the purity and authenticity of the bioactive isolates.

- **UV-Visible and Infrared (IR) Spectroscopy:** The techniques of UV-Visible and Infrared (IR) spectroscopy are also used in the identification of functional groups and major structural components of the isolated compounds. UV-Visible spectroscopy is used to identify characteristic absorbance peaks that are constituted by conjugated systems and chromophores and data provided by the literature show the right direction. The infrared spectroscopy also helps to identify individual functional groups like carbonyls, hydroxyls and olefinic bonds based on the distinct vibrational frequencies of the group. The structural assignments are confirmed by making a comparison of the UV and IR spectra with the standard reference data. This regulated method of isolation and characterization guarantees that the isolated bioactive compounds are structurally validated and give a good baseline on examining their structure-activity association, as well as elucidating the manner in which they act on their molecular mechanisms to bring about antidepressant actions.

Acute Toxicity Studies

Before investigating the pharmacological effects, it is essential to establish the safety profile of the plant extracts through standardized toxicity testing following international guidelines.

- **Study Design:** The research was conducted with the strict compliance with the Organization for Economic Co-Operation and Development (OECD) guideline 423, the Acute Toxic Class Method, to analyze the safety profile of the plant extracts. Wistar rats of the weight of 180- 200 grams were taken as experimental models (n=6), because they are the most widely used as they are physiologically relevant and have a standard response to toxicological testing. In order to maximize absorption and limit variability, the animals were fasted 12-16 hours prior to extracts administration, but free access to water was given

to the animals to avoid dehydration. Fasting was also continued another 3-4 hours after the dosage to prevent the possibility of food interfering with the absorption of the gastrointestinal system, thereby making the toxicity assessment reliable.

- **Treatment Protocol:** The therapy was carried out by use of plant extracts that were orally fed in 2000 mg/kg body weight dosage carried out by oral administration to allow exact dosing. The animals were closely monitored after the administration with regard to the manifestations of toxicity and abnormal behaviors. The most intense activities were observed in the initial 30 minutes, and constant observation was made to record any adverse reactions that may occur immediately. The intermittent though intensive observations were carried out throughout the next 4 hours, which is the most important time to observe acute toxic effects. The continued monitoring was conducted until 24 hours to detect any delayed reaction and a prolonged observation period of 14 days after the dose given to the subjects was used to determine any sub-acute/lingering toxic effects, which might affect the results of the safety test.
- **Parameters Evaluated:** A wide variety of parameters were tested to identify the presence of any toxic manifestations. Primary indicators of toxicity included mortality rate and the time of death, in case the latter occurred. To be able to identify neurotoxic effects, behavioral alteration like depression, excitation, aggression, and convulsions was observed. Systemic toxicity was observed to be indicated by autonomic symptoms, such as salivation, lacrimation, piloerection, urination and defecation. Neuromuscular effects were monitored by attentively noting changes in motor activity, ataxia, sedation, and hyperactivity. The integrity of the nervous system was tested by sensory reaction to touch, sound, and light, and the overall physical state of the animals was monitored, in order to detect any adverse changes or negative developments.
- **Ethical Considerations:** The research was done in accordance with the ethical standards, and the experimental protocol was approved by the Institutional Animal Ethics Committee as Approval No.GRKIST-

P/IAEC(M2)/2024/07. Each and every practice was in line with the National Institutes of Health (NIH) standards on treatment and use of laboratory animals, which treated the animals in a humane manner. Humanity endpoints were also followed to the letter with any animal exhibiting more than the necessary suffering euthanized to avoid suffering more than necessary. Also, the research used the fewest animals needed to yield statistically acceptable data to comply with the 3Rs principle of research ethics involving animals (Replacement, Reduction, Refinement).

- **Outcome Measures:** The outcome measures were to establish a median lethal dose (LD₅₀) or to classify the plant extracts in the global harmonized system (GHS) toxicity of toxicants. The results were vital as safety margin in such that, suitable therapeutic doses would be selected in further pharmacological assessments, which is usually taken as 1/10 or 1/5 of the maximum dose of non-lethal level discovered during the research. Moreover, any adverse effects that were detected were indeed well documented to develop a complete safety profile, which acts as a vital guide to future clinical practice, and an assurance that the plant extracts are effective and safe to be used in any therapeutic purpose.

Experimental Design for Pharmacological Studies

A comprehensive experimental design will be implemented to systematically evaluate and compare the antidepressant potential of both plants using established animal models and standardized protocols.

Animals:

1. Wistar rats (180-220g) obtained from the animal house Guru Ramdas Khalsa Institute of Science & Technology, Pharmacy Jabalpur.
1. Housing in polypropylene cages under controlled conditions:
 - Temperature: 25 ± 2°C
 - Relative humidity: 45-55%
 - 12/12 hour light/dark cycle
 - Standard laboratory diet and water ad libitum

2. Acclimatization period of one week before experimentation to reduce stress-related variables

Experimental Groups: Six treatment groups (n=6 per group) will be established:

1. Group I: Control (Normal saline, 0.01 mL/g) - To establish baseline behavioral responses
2. Group II: Standard (Imipramine, 15 mg/kg, i.p.) - As positive control for antidepressant activity
3. Group III: T1-AGG (*Withania sonnifera*, 20 mg/kg) - Low dose to establish dose- response relationship
4. Group IV: T2-AGG (*Withania sonnifera*, 40 mg/kg) - High dose to evaluate dose- dependent effects
5. Group V: T3-AGG + Imipramine (10 mg/kg each) - To assess potential synergistic effects
6. Group VI: *Piper longum* (200 mg/kg) - To evaluate comparable dose effects for comparison

Treatment Duration:

1. Acute studies: Single administration 60 minutes before behavioral testing
2. Chronic studies: Daily administration for 14 consecutive days with testing on the 14th day

Route of Administration: The route of administration was also chosen well in this study depending on the substances under test. Plant extracts and extract combinations were delivered orally by the gavage procedure, which precisely doses and resembles the natural pathway of their human intake and makes the results more translational. The standard reference drug, imipramine, on the other hand, was administered via intraperitoneal injection so that it was able to achieve rapid systemic introduction of the drug and to maintain a constant level of plasma concentration, which is essential in determining the pharmacological effectiveness of the plant-based treatments.

Blinding and Randomization: In order to maintain the integrity and validity of the experimental findings, the processes of blinding and randomization were strictly followed. Animals were assigned to different treatment groups randomly to avoid selection bias and to ensure that any effects that were witnessed could be due to the treatments and not differences that existed in the groups beforehand. The behavioral measurements were made blindly i.e. the observers were not aware of the allocation of treatments hence the observer bias was reduced to the maximum. Moreover, the rotation of the testing order was done in a systematic manner to cancel the effect of circadian rhythms that may influence the behavior of the animal as well as hence confound the results.

Study Phases: The experimental study had both the acute and chronic phase to give a holistic assessment of the treatment effects. The acute phase was dedicated to determining the effect of the single dose administration on the short-term effect, and it was possible to detect the effects of fast onset and acute toxicity. Conversely, the chronic phase entailed a long period of treatment of the treatments to ascertain their effectiveness and safety in the long-run. The two phases methodology played a critical role in determining the short term and long term therapeutic potential of the plant extracts and their combinations. This experimental design will facilitate a complete analysis of the antidepressant potential of the two plants and come up with their effectiveness, dose response, and possible use in combination therapy with the standard antidepressant medication.

In Vitro Studies

Biochemical studies will be performed in vitro to examine the biochemical pathways of antidepressant effect of the plant extracts and its bioactive components especially monoamine oxidase (MAO) inhibition.

MAO Inhibition Assays:

- **Preparation of Enzyme Source:** Enzyme sources used in the MAO inhibition studies were carefully prepared using male rats. The animals were killed humanely through cervical dislocation and the tissues of the brain were immediately removed on ice in order to preserve the integrity of the enzymes. To extract the mitochondrial enzymes in the most effective way the collected

brains were homogenized in 0.25 M of the solution of sucrose at a ratio of 10 volumes to be homogenized. The homogenate was centrifuged at 1000g at 10 minutes at 4 °C to remove cellular debris. This supernatant was further centrifuged at 16,000g at 30 minutes at 4 °C to give a mitochondrial pellet that contained MAO enzymes in abundance. This pellet was re-suspended in the solution of sucrose and kept at -80 °C until it was used again in assays of activities.

- **Enzyme Activity Measurement:** the monoamine oxidase (MAO) activity was measured using a sensitive fluorometric approach. The substrate used in this assay was kynuramine and when catalyzed by MAO, it is metabolized to form fluorescent 4- hydroxyquinoline. The reaction mixture was 50 μ L of the mitochondrial fraction (1mg /mL protein concentration), 50 μ L of kynuramine (0.1mM) and 50 μ L of test substance or control in 100 mM potassium phosphate buffer (pH 7.4). Enzymatic activity was allowed to take place by incubating the reaction at 37 °C over a 30 minute period, which was followed by the termination of the reaction using 100 μ L of 2N NaOH. The fluorescence produced was quantified with an excitation wavelength of 320 nm and with 380 nm as the emission wavelength, which gave a direct measure of the activity of MAO.
- **Determination of Inhibition Parameters:** The inhibitory properties of the plant extracts and isolated compounds on the MAO activity were determined at different concentrations (2-36 mM). The concentration necessary to cause an effect is expressed as IC₅₀ values. To compare the results, the use of standard MAO inhibitors was considered l-deprenyl, which is a selective MAO-B inhibitor, and clorgyline which is a selective MAO-A inhibitor both at the concentration of 1 mM. This enabled the effective running of a comparative analysis of the efficacy of the plant derived compounds with well-established pharmaceuticals.
- **Kinetic Analysis:** In order to understand how the inhibition occurs, kinetic experiments were carried out by determining the activity of enzyme in the presence of various concentrations of substrate in the presence or absence of the inhibitors. Plots of Lineweaver-Burk were plotted to obtain the kinetic parameters K_m (Michaelis constant) and V_{max} (maximum velocity). This discussion made it possible to classify the type of inhibition as either

competitive, non- competitive, or uncompetitive. Moreover, inhibition constants (K_i) were determined and it gave more information about the binding affinity and mechanism of action of the test substances.

- **Reversibility Studies:** The reversibility of the enzyme inhibition by the test compounds was studied using the dialysis experiment. The inhibitors were pre-incubated with mitochondria followed by dialysis of the mitochondria against potassium phosphate buffer over 4 hours at 4 °C to eliminate unbounded inhibitors. Measurement of MAO activity was again done at the end of dialysis and compared with the pre-dialysis level. A major reinstatement of activity following dialysis was an indication of reversible inhibition but permanent inhibition indicated an irreversible binding to the enzyme.
- **Selectivity Assessment:** Selectivity between the two isoforms of MAO-A and MAO-B was also established through the differential inhibition studies. The selective inhibition of MAO-B with l-deprenyl and MAO-A with clorgyline enabled each of the two isoforms to be evaluated independently regarding their inhibition by the test compounds. Upon these findings, based on the selective inhibitions, a selectivity index was determined to measure the selective inhibition of MAO-A over MAO-B. This selectivity plays a pivotal role in the foretelling of therapeutic capacity and side effect characteristics in the use of antidepressants.

Neurotransmitter Release Assays:

- **Synaptosome Preparation:** In preparation of the neurotransmitter release assays, male mice synaptosomes were carefully prepared using the cerebral cortex. Synaptic structures were maintained by care with brain tissues which were isolated and homogenized in an ice-cold 0.32 M shelf of sucrose. The process of homogenization was followed by a series of centrifugation. To retain the viability and functionality of the synaptosomes throughout the tests, the final synaptosomal pellet was resuspended in Krebs-Ringer solution at a physiological pH of 7.4.
- **Neurotransmitter Release Measurement:** In order to measure the release of neurotransmitters, the purified synaptosomes were preloaded with radiolabeled neurotransmitters: [^3H]- 5-HT (serotonin), [^3H] -NE

(norepinephrine), or [³H]-DA (dopamine). The preloading of the synaptosomes was done by incubating the synaptosome with the radiolabels in 15 minutes at 37 C to ensure that sufficient uptake of the neurotransmitters was done into the nerve terminals. The synaptosomes were then washed well to eliminate non- internalized radiolabeled molecules. The effects of the test compounds on the study were evaluated in terms of modulating the release of neurotransmitters by preincubating the synaptosomes with the test compound after washing with it over a period of 15 minutes. A 5-minute depolarization of the synaptosomes using 15 mM KCl was then used to stimulate the release of neurotransmitters. The radiolabeled neurotransmitters released were pooled by filtration then radiometrically measured by liquid scintillation counting giving exact values of the effect of test compounds on the dynamics of neurotransmitter release.

Such in vitro experiments will clarify the biochemical processes of the plant extracts and its constituents and their impact on monoamine metabolism and release of neurotransmitters, which are key areas of antidepressant effects.

In Vivo Behavioral Studies

The efficacy of the plant extracts as antidepressants in relation to conventional antidepressants will be of paramount importance through the behavioral studies which will use the well-established animal models of depression.

Forced Swim Test (FST):

1. **Apparatus and Conditions:** The Forced Swim Test (FST) is administered in a glass cylindrical container with a diameter of 20 cm and height of 50 cm which is filled to a depth of 30 cm with water. This arrangement is used to make sure that the rats cannot get away but also allows enough space that the rats could swim. To avoid the development of hypothermia, which may introduce another stress factor on the animals and confound the findings, water temperature is kept at 25 ± 2 C. The controlled environment plays a very important role in the process of making sure that any.
2. **Testing Procedure:** Rats are introduced in the cylinder filled with water one at a time and left to swim freely. This is a test which lasts 6 minutes, however, the actual time of behavioral analysis is 4 minutes since the first 2 minutes are

considered to be the habituation to the water environment. The main parameter measured during recording stage is the immobility time, which implies that the rat is floating with minimum movements, but only inappropriate movements to keep its head above the water. The test uses video recording to allow blind analysis which minimizes the possibility of observer bias and results in a better and more objective analysis of the animal behavior.

3. **Experimental Paradigms:** The FST can be conducted in two experimental paradigms. The acute test involves the administration of the test substance on the rats in a single dose of the test substance 60 minutes prior to the FST. This enables one to determine the immediate impacts of the substance on behavior. In the chronic test, the test substance is given in daily doses to the rats over 14 days and the FST is done on the 14th day, 60 minutes after the last dose. The chronic test is used in giving the insight on the long-term effects of the treatment on behavioral responses and this enables them to assess the potential antidepressant-like effects in the long-term.

Tail Suspension Test (TST):

1. **Apparatus and Setup:** Tail Suspension Test (TST) is performed by using a horizontal bar 50 cm above the ground. Adhesive tape is used to suspend the rats by their tails thereby letting them freely swing in the air. The test is conducted in a testing chamber with dimensions of 25x 25x 30cm where the behavior of the rat can be viewed in a controlled environment. This arrangement is meant to cause behavioral reactions that are indicative of depressive-like symptoms, which are further applied in assessing the actions of the test substance.
2. **Testing Procedure:** The rat is held in the air in the testing chamber in total of 6 minutes with the tail held in the air. The attention of behavioral observations is concentrated on the final 4 minutes of this time during which the main parameter of interest is the immobility time. The lack of voluntary movement is referred to as immobility, whereby the rat is still and suspended; immobility is usually employed as an aspect of coping behavior, when the rat feels that it lacks escape routes.

3. **Experimental Paradigms:** There are two experimental paradigms of the TST. A single dose of the test substance is used in the acute test on the rats 60 minutes prior to the TST. This design enables one to observe immediate behavioral effect after administering the compound once. In chronic test, the rats are treated on the same with the test substance on a daily basis in 14 days, and the TST is performed at the 14 th day, 60 minutes after the last dose. The paradigm of chronic administration considers the effects of the substance in the long term, and this is what can give details about the possibility of it acting as an antidepressant in the long-term and which changes over repetitive exposures.

Open Field Test (OFT):

1. **Apparatus:** Open Field Test (OFT) is done through a circular arena which has a diameter of 80 cm. There are three concentric circles which subdivide the floor of the arena into 36 equal parts. The design enables the exact quantification of the movements and behavior of the rat, thus providing a clear structure into the measurement of the locomotor and exploratory activities of the rat in a specified area. The arena is normally located in a room with a lot of light, and as distractions are minimized there are no distractions in the test.
2. **Testing Procedure:** In the case of the OFT all the rats are put in the middle of the arena so that the test can commence. Subsequently, the rats are allowed to explore freely within a time of 5 minutes whereby their natural behavior is noted. The most important parameters are the locomotor activity (measured as the number of segments crossed by the rat in the course of the test), and the exploratory behavior (measured by observing such behaviors as rearing and grooming). The tests are administered 60 minutes following administration of the drug to give the drug time to work and probably affect the behavior of the animal.
3. **Purpose:** The main aim of Open Field Test is to distinguish the effects of certain antidepressants on locomotor activity and general locomotor activity. Although the Forced Swim Test (FST) and the Tail Suspension Test (TST) assess depressive-type behaviors, the OFT makes certain that the depressive-like behavioral results of the awarded tests are caused not only by the raised level of activity, but also the raised psychostimulant activity. Through the

evaluation of locomotion and exploration within a controlled setting, the OFT also adds more data on behavioral characteristics of the drug.

Reserpine-Induced Depression Model:

1. **Induction of Depression-like State:** Reserpine-Induced Depression Model is the application of reserpine at a dosage of 4mg/kg to induce depletion of monoamine neurotransmitters (especially serotonin, norepinephrine, and dopamine). This drainage simulates the symptom of depression in human beings, which results in various behavioral modifications in animals. The typical effects caused by reserpine are ptosis (eyelid drooping), catatonia (muscular inflexibility and immobility), and sedation (loss of responsiveness). These symptoms are strictly observed since they are indicators of neurochemical variations with depression conditions.
2. **Treatment Protocol:** After induction of the depression-like condition using reserpine, test substances are administered to determine the capacity of the substance to reverse the symptoms. The efficacy of the treatments will be determined at certain times (1, 2, 3, and 4 hours of the administration). This enables the determination of any changes in the cases of depression-like symptoms as time increases and the presence and persistence of the therapeutic effects of the test substances.
3. **Assessment Parameters:** The evaluation of the depression-like symptoms is conducted through the measure of a series of behaviors. Ptosis is rated on a 0-3 scale where 0 indicates no ptosis whereas 3 indicates total eyelid cover. The Catatonia condition is determined through muscular rigidity and immobility as the degree of immobility and lack of voluntary movement. Sedation is assessed through decreased responsiveness of the rat to external stimuli e.g. touch or noise. The blinded observers make all observations in order to limit bias and measure the treatment effects objectively.

Such behavioral paradigms will give a thorough evaluation of the antidepressant-like properties of the plant extracts, and this will set the effectiveness of the extracts in acute and long-term treatment paradigms of antidepressant-like substances.

Biochemical Analysis

Biochemical analyses will be conducted to investigate the neurochemical mechanisms underlying the antidepressant effects of the plant extracts and their bioactive constituents.

- **Brain Tissue Preparation:** The biochemical experiment commences with the sacrifice of animals by cervical dislocation after behavioral tests are done. The brain is quickly dissected on ice after sacrifice so that neurochemical compounds are not destroyed as much. The isolated regions of the brain are then analyzed such as the striatum, hippocampus, and cerebral cortex. Of special relevance is the striatum with its dopaminergic terminals, which participate in motivation and reward. The emotional regulation and the stress response are fundamentally dependent on the hippocampus, whereas the serotonergic and noradrenergic projections are located in the cerebral cortex and are essential to regulate the mood. This isolated tissue is brought to a homogenate in the presence of 0.1 M perchloric acid which has 0.1 mM EDTA to avoid degradation of neurotransmitters. Homogenization is followed by centrifugation of the sample at 14,000g at 4 °C to get a clear supernatant which is followed by analysis of the neurotransmitters.
- **Neurotransmitter Level Determination:** The determination of the neurotransmitter levels within the brain tissue is done using high-performance liquid chromatography (HPLC). The neurotransmitters of interest are then partitioned on relevant columns and the detection done on the column either through the electrochemical or fluorometric detection system depending on the neurotransmitter of interest. All quantification is done by comparison against the standard curves made of known concentrations of the neurotransmitters. This provides the ability to accurately measure the levels of neurotransmitters of the brain areas being studied.
- **Neurotransmitters Measured:** Biochemical analysis is done on three major neurotransmitters (serotonin (5-HT), norepinephrine (NE), and dopamine (DA)). Serotonin plays an important role in mood regulation and is usually found to be out of control during depression. Norepinephrine participates in arousal, attention and stress response, whereas dopamine has a major role of

motivation, reward and pleasure. These neurotransmitters are chosen depending on their role in the pathophysiology of the depression and their possible role in the action of the plant extracts under study.

- **Parameters Calculated:** based on the HPLC results the absolute concentrations of the neurotransmitters are calculated in units of ng/g of tissue. Also, the percentages of neurotransmitters changes are determined against the level of the control group to determine the effects of the treatments. In reserpine trained animals, which is a monoamine depleting drug, the recovery of the neurotransmitter levels is especially significant to assess whether the plant extracts were effective in restoring the depressed like condition. These analyses will give information on the neurochemical action of the plant extracts and the bioactive constituents.
- **Correlation Analysis:** Correlation Analysis is done in order to gain further insights regarding the relationship between the level of neurotransmitters and the behavioral results. The way in which the variation in neurotransmitter levels is associated with the behavioral parameters recorded in the animal models will be discussed in relation to the findings of the Forced Swim Test (FST) and Tail Suspension Test (TST). Neurochemical profiles of the various treatment groups will also be compared to determine the relative efficacy of each treatment and its effect on the systems of neurotransmitters in depression. This is the detailed analysis that will assist in explaining neurochemical mechanisms through which the extracts of the plants have their antidepressant effects.

Statistical Analysis

Rigorous statistical analyses will be employed to interpret the experimental data and draw valid conclusions about the comparative antidepressant effects of the plant extracts.

- **Data Organization and Preprocessing:** The pre-processing and organization of the experimental data will be used to start the statistical analysis. These include computing means and standard errors of the mean (SEM) of all the measured parameters, and ascertaining that the data is well summarized and variability represented. To test whether the data are normally distributed,

Shapiro-Wilk or Kolmogorov-Smirnov tests will be used to test the normality. In case the data fail to satisfy the assumptions of the parametric tests, the transformation of the data will be done accordingly to normalize it. It will also identify and deal with the outliers relying on the set statistical parameters to avoid the distortion of the results.

- **Primary Statistical Analyses:** A statistical method to be employed in this research will be one- way Analysis of Variance (ANOVA) in order to compare several. This will assist in establishing whether there exist any significant differences among groups. F-statistics will be done and a level of $p=0.05$ will be established to find out the statistical significance of treatment effects. In case the ANOVA results reflect the existence of significant differences, the post-hoc test of Tukey will be used to demonstrate particular between-group differences. The test will also adjust various comparisons to regulate the rate of Type I error and will produce confidence intervals of the differences in the means.
- **Two-way ANOVA:** Two-way ANOVA will be performed in order to analyze time-course data. This will be done by analyzing the primary impacts of the treatment, time, and their interaction on the outcome variables. It will decompose the variance and identify the proportion of contribution of each factor and give information on the change in the effects of treatment with time. Should the two way ANOVA results be significant, the post-tests will be corrected through Bonferonni to balance the effect of the number of comparisons carried out such that the error margin does not go out of hand.
- **Additional Analyses:** To further understand the relationship between dose and response then regression analysis will be carried out. This will be useful in determining a dose- response curve and determination of ED50 value which is a dose producing 50 percent of the maximum effect. Also, the correlation analysis will be performed based on the Pearson or Spearman correlation coefficients with a view of the investigation of the relationship between the behavioral and biochemical parameters. These correlations will be statistically compared to determine whether there are any mechanistic relationships between the levels of neurotransmitters and the behavior observed.
- **Comparison with Standard Drug:** The effectiveness of the plant extracts will also be established in relation to a standard antidepressant imipramine

drug. The comparison of relative efficacy will be done by calculating the effect sizes which measures how large the treatment effects are in relation to the standard drug. This analogy will offer significant background to the research on the strength of the plant extracts and its possible use in treating depression as an alternative.

- **Software and Presentation:** The statistical analysis will all be done on GraphPad Prism 8.0 (GraphPad Software, Inc., USA), a powerful software package based on scientific analysis of data. Presentation will be in the form of mean and standard error of the mean in tables and figures with statistical significance clearly set in form of asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) for easy interpretation. Data will be visually represented using appropriate graphical formats such as bar graphs, line charts, or scatter plots, depending on the nature of the data and the comparisons being made. This approach will ensure clarity and precision in presenting the results.

This comprehensive statistical approach will allow for a rigorous analysis of the experimental data, providing reliable insights into the comparative antidepressant efficacy and mechanisms of *Piper longum* and *Withania sonnifera*.

Confirmation of Statistical Tests Used

To clarify the statistical analysis approach adopted in the present study, the following methods were employed:

1. **One-way Analysis of Variance (ANOVA)** was used as the primary statistical test to compare multiple treatment groups at each experimental time point, including both acute and chronic studies. This analysis was performed to determine the presence of statistically significant differences among the treatment groups.
2. **Post-hoc analysis:** When the one-way ANOVA indicated a statistically significant difference ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) test was applied for multiple comparisons. This post-hoc test was used to identify specific between-group differences while minimizing the risk of type I error.
3. **Two-way ANOVA** was employed for the analysis of time-course data, such

as reserpine-induced behavioral symptoms recorded at 1, 2, 3, and 4 hours. This analysis allowed assessment of the main effect of treatment, the main effect of time, and the interaction between treatment and time (treatment $\times$ time).

4. **Levels of statistical significance** were denoted as follows:

- $p < 0.05$ — significant
- $** p < 0.01$ — highly significant
- $*** p < 0.001$ — very highly significant

All statistical analyses were carried out using GraphPad Prism version 8.0 software. The experimental data were expressed as mean $\pm$ SEM (Standard Error of the Mean).

Comparative Analysis

A systematic comparative analysis will be conducted to evaluate the relative merits of *Piper longum* and *Withania sonnifera* as potential antidepressant agents.

Efficacy Comparison: A detailed comparison of the efficacy will be conducted to determine the antidepressive effects of *Piper longum* and *Withania sonnifera* in comparison with each other and in comparison to the conventional antidepressant, imipramine. This will involve a direct comparison of percentage time reduction in immobility of Forced Swim Test (FST), and Tail Suspension Test (TST), which is both a common measurement of the depressive-like behavior. The pattern of these effects in various experimental paradigms will be analyzed to determine the credibility of the antidepressant effect of the plant extracts. The acute and chronic effects will also be compared by looking at the beginning of the action of the antidepressants, the maintenance of the effects when the treatments are used long-term and the possibility of tolerance to the treatments. The plant extracts will also be compared in the various aspects of depression such as behavioral despair (through FST and TST), anhedonia (through variation in the levels of neurotransmitters) and reserpine induced symptoms, including ptosis, catatonia and sedation.

Mechanistic Comparison: The mechanistic comparison will investigate the main mechanism of action of *Piper longum* and *Withania sonnifera*. This will involve a

study of their profiles of monoamine oxidase (MAO) inhibition that will involve their selectivity, potency, and reversibility. The induced influence on the level of the monoamine neurotransmitters, such as serotonin, norepinephrine, and dopamine, will also be compared as well, and these neurotransmitters are also vital in controlling mood. It will also be discussed whether other neurotransmitters systems, including the GABAergic or glutamatergic systems, are involved or not. The temporal nature of neurochemical effects will be considered, including the nature of whether biochemical effects are instantaneous, delayed or not how the effects are interrelated with behavioral effects that have been observed. The continuity of the neurochemical changes will also be measured to establish how long the impact of the antidepressants will last. In addition, the mechanism of these plant extracts will be compared to the known antidepressants, including monoamine reuptake inhibitors, to identify similarities, differences, and new mechanisms unique within the plant extracts.

Dose-Response Relationships: Dose-response relationships will be determined of both the *Piper longum* and *Withania sonnifera* with the aim of determining the best dosage range of each plant extract. This will involve the determination of the minimum effective doses, doses that bring about maximum effects and the therapeutic index (ratio of the toxic dose to the effective dose). This will be done by analyzing the dose-response curves and determining how steep the curves are, whether they have ceiling effects or the bell shaped responses, as well as whether dosage changes might be required in clinical application. The data shall be very important in setting safe and effective therapeutic regimen as they will give details on how the plants should be dosed.

Safety Profile Assessment: Comparison of *Piper longum* and *Withania sonnifera* safety profile will be carried out with emphasis on the potential side effects. The results of acute toxicity will be analyzed to identify the levels of safety of each plant extract. The treatments will be evaluated in terms of their effects on normal locomotor activity to determine whether the treatments lead to motor dysfunction or sedation. Their side effects may occur because of the mechanisms of action of the plant extracts, and thus they will be assessed on the effects of these extracts on the neurotransmitters systems. The risk benefit analysis will be done based on the therapeutic index of the effective dose to the toxic dose, safety benefits of the antidepressant compared to the conventional antidepressants and the contraindications or drug interactions that may

occur when these extracts are used concomitantly.

Potential for Combination Therapy: It will explore the possibility of using *Withania sonnifera* with other antidepressants to determine any synergistic effects. This also involves assessing whether the combination can be used to reduce dose of the traditional drug whilst still being effective, and also determine the mechanistic explanation of the observed synergism (complementary pathways in which antidepressant effects are mediated). They will also explore the possibility of combining *Piper longum* and *Withania sonnifera* with the aim of whether their various mechanisms of actions can be used to complement one another. The proportion of these extracts that provides the greatest efficacy will be identified and the safety profile of the combination established compared to the safety profile of single treatments. Clinical implications of the combination therapy will be taken into attention, such as possible ways of application in therapeutically nonresponsive depression, methods of minimizing side effects of traditional antidepressants and whether there is any possibility of increasing the therapeutic effect sooner.

Practical Applications: Practically, the study will be based on the formulation factors including the optimal methods of extracting the active ingredients of *Piper longum* and *Withania sonnifera*. The best dosage rates of bioavailability, both with traditional Ayurvedic formulations and modern formulations will also be addressed. Also, the stability and shelf-life of the active constituents shall be determined to verify the long-term effectiveness of these treatments. The therapeutic potential of the plant extracts will be discussed in relation to the cases of particular types of depression, such as major depressive disorder, dysthymia, as well as persistent depressive disorder. The applicability of these extracts to comorbid anxiety and depression will be also taken into consideration as they may serve to cover several aspects of mental health. The differences between *Piper longum* and *Withania sonnifera* and the traditional antidepressants will be emphasized: *Piper longum* and *Withania sonnifera* have fewer side effects, a quicker action, and a multi-target effect.

Traditional Medicine Context: The Ayurvedic medicine of *Piper longum* and *Withania sonnifera* will be subjected in terms of its traditional uses and compared with the experimental data to confirm their use in depression treatment. The traditional preparation methodology will be explained scientifically and will help

reconcile the fallacy between the ancient practices and the knowledge regarding scientific practices. Agreeability with traditional dosing recommendations will be questioned as well. Moreover, the possibility to apply these extracts of plants in the contemporary psychiatry will be examined, and the use of these extracts may be both a single-agent treatment of mild to moderate depressive disorder and a supplementary treatment to traditional antidepressants. Such plants can also be used in prevention efforts of vulnerable populations and this expands their therapeutic range even more. This comparative systematic review will present a holistic assessment of *Piper longum* and *Withania sonnifera*, its effectiveness, action mechanism, safety risks, and application in depression treatment.

3.11 Plant Profile

Piper longum Botanical Description

Piper longum L. (Family: Piperaceae), commonly known as long pepper or "Pippali" in Ayurveda, is slender aromatic perennial climber with woody roots & numerous creeping, jointed stems that are thickened at nodes. Plant grows to a height of 1-2 meters with spread of 0.5-1 meter.

- **Leaves:** Simple, alternate, 5-10 cm long & 3-5 cm broad, ovate, cordate with an acuminate apex, dark green above and pale beneath with distinct veins.
- **Flowers:** Minute, monoecious or dioeciously, arranged in solitary, cylindrical, peduncled spikes; female spikes are 2-3 cm long, while male spikes are slender & up to 5 cm long.
- **Fruits:** Small, ovoid berries, initially green and turning blackish-red or dark gray upon ripening, tightly packed in elongated, cylindrical fruiting spikes measuring 2.5-5 cm in length and 0.4-0.7 cm in diameter.
- **Seeds:** Small, globular, and black.

Geographical Distribution

Piper longum is found in Indo-Malayan region, primarily in hot regions of India, Sri Lanka, Malaysia, and Indonesia. And in India, it occurs in the wild and as cultivated species in Eastern Himalayas; Khasi & Mikir hills; lower hills of Bengal; Assam,

Western Ghats; & in evergreen forests of Tamil Nadu & Kerala. It is grown on commercial scale in Maharashtra, Assam, West Bengal, Kerala and Tamil Nadu. Plant grows well in tropical & subtropical conditions of high humidity & moderate temperature (25-30°C) under partial shade with well-drained fertile, organic matter rich sandy loams. It occurs at sea level up to 1,500 meters.

Traditional Uses

Ayurvedic Medicine: *Piper longum* or Pippali is an essential medicinal herb that has been used since over three thousand years in Ayurveda. It is also one of the major components of the classical Trikatu which is considered to improve the bioavailability and the therapeutic performance of other herbs. Pippali is originally administered in the respiratory diseases like cough, bronchitis, asthma, and tuberculosis. It is also widely used in the management of digestive ailments including dyspepsia, flatulence, and abdominal pain. Besides, it is also suggested in metabolic disorders especially diabetes and obesity. Ayurvedic texts describe *Piper longum* as a rejuvenative (*Rasayana*) and aphrodisiac (*Vajikarana*) drug, and it is also employed in the treatment of neurological conditions such as paralysis, epilepsy, and depression.

Traditional Chinese Medicine: *Piper longum* is the Traditional Chinese Medicine (TCM) referred to as Bi Bo. It is mainly applied in the treatment of gastric disorders, cold extremity and poor peripheral circulation. The drug is also prescribed for respiratory complaints including asthma, cough, and bronchitis. Furthermore, it is used to relieve pain and inflammation, especially in conditions associated with arthritic disorders.

Folk and Ethnomedicine: In various folk medicine practices, *Piper longum* has been used as an antidote against snake and scorpion venom. It is used as a topical agent to treat muscle pain and inflammation as well as headache. It is also used in the management of menstrual disorders and enrichment of lactation among lactating mothers by the traditional healers. It is also applied as a natural remedy of insomnia, anxiety, and depressive disorders

Phytochemical Constituents

Piper longum is a source of a wide variety of bioactive phytochemicals, the most

typical of which constitute alkaloids and amides. The pungent taste and numerous pharmacological effects of the plant are due to the major alkaloid, piperine. Other important alkaloids and amides include piperlongumine, piperlonguminine, piperonaline, piperundecalidine, piperderidine, retrofractamide A, and pellitorine. The plant also contains volatile oils (1–2.5%) such as caryophyllene, piperonal, zingiberene, germacrene D, limonene, bisabolene, and sylvestrene. Lignans like sesamin, pulvialtol, fargesin, and eudesmin have also been reported. In addition, *Piper longum* contains other constituents including piperettine, piperolein B, piperchabamide D, steroids such as β -sitosterol and stigmasterol, along with polysaccharides, proteins, and amino acids.

Pharmacological Properties

Extensive laboratory and clinical researches have proved that *Piper longum* has a wide range of pharmacological actions. It exhibits powerful antidepressant effect via inhibition of monoamine oxidase (especially, MAO-B), regulation of serotonin, norepinephrine and dopamine, alleviation of immobility of the forced swim test and tail suspension test and reversion of Gyrind against reserpine-induced behavioral defects. The plant also has significant immunomodulatory properties, whereby it boosts cellular and humoral immunity, raises macrophage phagocytic capacity, antibody titer and delayed type hypersensitivity. The effects of the compound include anti-inflammatory and analgesic, which are mediated by the inhibition of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α), inhibition of the formation of prostaglandins via the action of COX-2, inhibition of the activation of NF- κ B, and antioxidant free-radical-scavenging activities.

Moreover, *Piper longum* has positive implications to the digestive system by increasing the secretion of digestive enzymes, enhancing the gastrointestinal motility, guarding the liver against toxins and inhibiting the development of peptic ulcers. It is a bronchodilator, expectorant, mucolytic, and prevents the release of histamine by the mast cells in the respiratory system. Other pharmacological actions are antimicrobial, antiparasitic (especially antimalarial), antidiabetic, neuroprotective, and bioavailability-enhancing activity and this has facilitated its extensive application in traditional and modern medicine.

Toxicological Studies

Piper longum has been considered to be relatively harmless in recommended dosage. Acute toxicity studies revealed that the LD₅₀ of ethanol extract is greater than 2,000 mg/kg body weight in rodents, demonstrating wide safety margin. LD₅₀ has been reported to be safest for piperine, which is more than 100 mg/kg. There are no reports on severe side effects at therapeutic doses used in traditional medicine. Nevertheless excessive consumption of high doses can induce gastric irritation, hepatic enzyme increase, & eventually reproductive toxicity. Use is not recommended in pregnancy or lactation owing to inadequate safety data in these groups.

Withania somniferous Botanical Description

Withania somniferous (L.) Dunal (Family: Solanaceae), commonly known as Ashwagandha or Indian Winter Cherry, is erect, branching, perennial under shrub up to 30-150 cm high. Having smell like horse ("Ashwa" meaning horse & "gandha" meaning smell in Sanskrit.)

- **Stems:** Erect, branched, terete, solid, densely covered with fine star-shaped (stellate) hairs, giving them grayish appearance.
- **Leaves:** Simple, ovate to elliptic, 5-10 cm long and 2.5-5 cm wide, with entire margins, alternate, estipulate, petiolate, soft, and covered with fine woolly hairs.
- **Flowers:** Small, greenish-yellow or pale green, bell-shaped, maxillary, solitary or in few-flowered clusters, pentamerous, actinomorphic, and hermaphrodite.
- **Fruits:** Globose berries, 5-8 mm in diameter, enclosed in persistent calyx, green when unripe & orange-red when mature.
- **Seeds:** Numerous, yellow to light brown, deiform (kidney-shaped), embedded in mucilaginous pulp.
- **Roots:** Stout, fleshy, cylindrical, whitish-brown, 30-40 cm long & 1-2 cm in diameter, with a characteristic horse-like odor.

Geographical Distribution

Withania sonnifera, called Ashwagandha is an important medicinal plant of high commercial value, known to occupy ecological niches in several agro-climatic & soil types. Originating in the Middle East & Mediterranean, it is particularly prevalent in the Indian subcontinent, where it is an integral part of Ayurvedic medicine. It has also become naturalized in regions of Africa, Europe, and the Americas and is currently grown, primarily for its medicinal uses. Location we find *Withania sonnifera* in the drier regions of India, doing particularly well in states like Madhya Pradesh, Rajasthan, Punjab, Gujarat,

Maharashtra, Uttar Pradesh and Himachal Pradesh. of these, the regions that have been mentioned to be potentially natural are Madhya Pradesh, Gujarat, Maharashtra and Rajasthan, also known for its wide practice of commercial cultivation of the plant. These states have become the major grower States in India because of the conducive agro-climatic conditions and advanced agricultural practices for growing Ashwagandha. In these areas, agriculture- based communities are using *Withania sonnifera* in their agricultural systems as an agro- practice, for local herbal use with a view to serving to meet increasing needs of herbal and pharmaceutical industries. The ecological flexibility of the plant is one of the reasons for its wide distribution. It thrives in a dry or semi-arid climate and can be farmed where average rainfall is low and up to moderate levels. Sandy or loamy soils that can be well drained are the preferred location of *Withania sonnifera* to avoid having a waterlogged root system. Its optimum soil pH for growth is between 7.5 and 8.0, slightly alkaline in nature, which is available in widespread agro-ecological zones of India. It is a very drought-tolerant crop which can also be grown in rain-fed and sustainable production systems, particularly in areas suffering from water scarcity, and it requires very little water after establishment. The optimal temperature of the plant could be 20 degrees to 35 degrees Celsius, yet it is able to adapt to much wider temperature ranges. This temperature ranges are consistent with the typical climate profiles of the regions which is most commonly cultivated. Furthermore, *Withania sonnifera* can be cultivated at elevations ranging from sea level to about 1,500 m above sea level, enabling it to be grown in the plains and hilly regions as well. This elevational range flexibility also favors its distribution over different regions of Indian subcontinent and beyond. Internationally, the growing popularity of natural and complementary

medicine practices has driven introduction and spread of the plant in non-native regions such as US, South America and Africa. In these areas, R&D activities are concentrated on the optimization of growing practices and the increase of yields in order to match the increasing global demand. Its medicinal value, tolerance to abiotic stresses and low maintenance due to less input requirements make AS a potential herb to be grown as crop in marginal and underprivileged conditions all across the globe

Traditional Uses

Withania somniferous has been one of the most revered medicinal plants in Ayurveda for over 3,000 years and is also widely used in Unani, Siddha, and various folk medicine traditions. In **Ayurvedic medicine**, it is classified as a “Rasayana” (rejuvenative) herb, known for promoting longevity, vitality, and overall well-being. It is used as an adaptogen to enhance resistance to stress and anxiety and is commonly prescribed for general debility, nervous exhaustion, and memory enhancement. The plant also finds its use in the treatment of arthritis, inflammation and rheumatic disorders, reproductive health including male fertility and female reproductive disorders. Moreover, *Withania sonmifera* is applied in the treatment of neurodegenerative conditions and mental diseases.

In **Unani medicine**, *Withania somniferous* is praised due to its tonic properties in general, especially the force of the body muscles and the increase of body mass. It is prescribed to treat the nervous system disorders such as Parkinson disease and epilepsy and is commonly used with the treatment of male sexual dysfunction as an aphrodisiac. Topically, it is used in the treatment of ulcers, carbuncles, and other skin related problems. In **folk medicine**, the plant is traditionally used as a sedative for the management of insomnia and anxiety. It is applied as a poultice for inflammations, ulcers, and painful swellings and is employed in the treatment of bronchitis, asthma, and tuberculosis. The herb is also used to support postpartum health, including enhancement of lactation in nursing mothers, and is traditionally applied in the management of mood disorders, including depression.

Phytochemical Constituents

Withania somniferous contains more than 80 bioactive compounds, with withanolides (steroidal lactones) being the characteristic and most pharmacologically significant

constituents. The **withanolides** include Withaferin A, which is the most extensively studied compound, along with Withanolide A, B, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, and S. Other related compounds such as withasomnine, withasomniferols A, B, and C, withasomidienone, withastramonolide, withanone, 27-deoxywithaferin A, and 17-hydroxy withaferin A have also been identified. The plant also contains several **alkaloids**, including somniferine, somnine, somniferinine, withanine, withananine, pseudo-withanine, tropine, pseudo-tropine, cuscohygrine, anferine, and anahygrine. In addition, **saponins** such as sitoindosides VII, VIII, IX, and X, as well as withanosides IV, V, and VI, are present. Other constituents include steroidal compounds such as β -sitosterol, stigmasterol, and diosgenin; flavonoids like quercetin, rutin, and isopelletierine; amino acids including alanine, aspartic acid, glycine, proline, tyrosine, and tryptophan; minerals such as iron, calcium, and phosphorus; and essential nutrients including carbohydrates, proteins, and dietary fiber.

Pharmacological Properties

Modern pharmacological studies have validated many of the traditional uses of *Withania somniferous* and have revealed a wide range of biological activities. Its **adaptogenic and anti-stress activities** are credited to the hypothalamic-pituitary-adrenal (HPA) axis modulation, which results in cortisol levels and stress hormone imbalances are normalized. The plant has been communicated to inhibit stress-related gastric ulcers, hypertension, and immunosuppression besides increasing resistance to physical, chemical, and biological stress. *Withania somniferous* also exhibits significant **antidepressant and anxiolytic activities**, mediated by the adjustment of serotonergic, GABAergic and dopaminergic neurotransmitter systems. Experimental research has shown a decrease in the duration of immobility in tail suspension test and forced swim test, inhibition of depressive symptoms caused by reserpine, alleviation of anxiety in other behavioral paradigm, and restoration of stress induced changes in neurochemical activity in the brain.

The plant shows notable **immunomodulatory activity**, associated with improvements of cellular immunity by the activation of lymphocytes, macrophages, and natural killer cells and promotion of antibody synthesis and humoral immunity. Its anti-inflammatory actions are realized by blocking NF-kB signaling and pro-

inflammatory cytokines, regulation of Th1/Th2 ratio and cytokine synthesis. In terms of **neuroprotective activity**, *Withania somniferous* defends against neurodegeneration in experimental animal models of Parkinson and Alzheimer disease. It preserves cognitive functions, decreases the deposition of β 2 amyloid and hyperphosphorylation of tau, prevents oxidative processes in the brain by stimulating glutathione levels in the brain and decreasing lipid peroxidation. Neuroregeneration as well as synaptic reconstruction is also stimulated by the plant.

The herb exerts important **endocrine system effects**, with control of thyroid activity and levels of thyroid hormone, control of insulin secretion and glucose metabolism, control over reproductive hormones and sex activity, and control over growth hormone and insulin-like growth factor-1 (IGF-1) signaling. Additionally, *Withania somniferous* demonstrates **cardioprotective and metabolic activities**, including cholesterol and triglyceride and ischemia reperfusion injury prevention in the heart, cardiac function in cardiomyopathy models, antihypertension effects (vasodilation and calcium channel) and antidiabetic effects (increased insulin sensitivity and glucose metabolism). Lastly, the plant has great anticancer effects, such as preventing angiogenesis and tumor metastasis, decreasing inflammation and oxidative stress in the tumor microenvironment, combining with conventional chemotherapeutic drugs, and chemoprevention effects that have been shown in different carcinogenesis animal models.

Toxicological Studies

Preclinical and clinical studies with *Withania sonmifera* have proven that it is safe and well tolerated. Acute toxicity studies revealed the lethal dose (LD_{50}) value more than 2000mg/kg body weight for methanolic extract, suggesting for safety margin. In clinical trials, doses of 1000 mg/day of standardized root extract have been well-tolerated with low toxicity. Side effects are reported as mild and rare, which include gastrointestinal upset, drowsiness and mild liver enzyme increases in huge amounts. However, women should avoid use during pregnancy as higher doses may be abortifacient. It can also interact with some medicines such as sedatives, thyroid medication, and immunosuppressants, so anyone taking these with an herbal dose would need to be watched carefully.

3.12 Statistical Analysis

All experimental data were expressed as Mean $\pm$ SEM (Standard Error of Mean). Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., USA).

Statistical Tests Applied:

1. **One-way ANOVA:** Used for comparing multiple treatment groups at single time point
 - Post-hoc test: Tukey's test for pairwise comparisons
 - Significance level: $p < 0.05$
2. **Two-way ANOVA:** Used for analyzing time-course data with repeated measurements
 - Factors: Treatment and Time
 - Post-hoc test: Bonferroni correction for multiple comparisons

Significance Levels:

- * $p < 0.05$ (significant)
- ** $p < 0.01$ (highly significant)
- *** $p < 0.001$ (very highly significant)

Results were considered statistically significant when p-value was less than 0.05.

3.13 Ethical Consideration

Current study was authorized by Guru Ramdas Khalsa Institute of Science & Technology, Pharmacy Jabalpur. Animal Ethics Committee (Approval No GRKIST-P/IAEC(M2)/2024/07) and carried out strictly in compliance with National Institutes of Health's (NIH) guidelines for animal use & care. Welfare of animals used in study during entire investigation was strictly respected, only minimum number of animals was employed in order to minimize animal suffering. Process started with the

establishment of a step-by- step research protocol, which was submitted to the committee and provided detailed rationale of the study objectives, methods, and anticipated results. Study was closely analyzed by the committee with regard to the scientific value of the research, not only of the investigational benefits but also of animal use in light of its consequences. They also reviewed animal welfare guidelines to make sure that experiment was in line with highest ethical standards. Following approval of protocol, experiments were performed according to stringent guidelines of the methods approved to protect welfare of animals used. Study was planned with stringent efforts to reduce pain and discomfort of the animals.

The animals were managed by trained staff during the investigations and the level of physical and psychological distress was kept to a minimum. The trialist would always control the dose and mode of administration of the test substance, and this dosing and administration were meticulously chosen for the minimum risk possible. Before any injections were made, numerous trials were conducted to establish the optimal doses and modes of application to minimize nonspecific pain or discomfort. The animals were also closely monitored for their health as well as for their welfare on a routine basis during the experimental period. This monitoring enabled early recognition of discomfort, distress or health problems that were immediately dealt with using appropriate interventions.

CHAPTER 4

ANALYSIS AND DATA INTERPRETATION

This chapter reports in full on the findings of the experiment involving both plants and the antidepressant activity. For *Piper longum*, the chapter deals with its MAO inhibitory potential and especially with the competitive and reversible inhibition displayed by piperine against both MAO-A and B. In case of *Withania sonnifera*, it reviews acute and chronic effects in behavioral animal models and its potential to counteract reserpine-induced symptoms such as ptosis, catatonia and sedation. The chapter closes with a comparative overview of the antidepressant mechanisms and efficacy profiles of the two plants.

4.1 Analysis and Data Interpretation

4.1.1 Analysis for *Piper longum*

Present study was carried out to screen for antidepressant natural MAO (monoamine oxidase) inhibitors from *Piper longum*, well-known ethnomedicinal plant. These MAO inhibitors work to prevent breakdown of key neurotransmitters in mood (dopamine, serotonin, nor epinephrine). Ethanol Fruit extract from *Piper longum* was examined for possible inhibition of MAO, especially from mouse brain. The inhibition was found to be significant, and thus it could be considered that the extract may be used as a natural antidepressant. Ethanol extract was subjected to bioassay-guided partition to afford their active MAO inhibition constituents. Bioassay-directed isolation studies were carried out to deduce the active ingredient in the fraction. Following such approach, the most relevant bioactive compound resulted piperine, the best known piperidine alkaloid which showed a potent MAO inhibition.

The chemical structure of piperine was confirmed through complete physicochemical and spectroscopic characterization, including:

- **Melting point determination:** to verify the compound's identity and purity.
- **Ultraviolet (UV) and infrared (IR) spectroscopy:** For analyzing functional groups and molecular structure.

- **Mass spectrometry (MS):** to verify molecular weight and composition of piperine.
- **Proton nuclear magnetic resonance ($^1\text{H-NMR}$) & carbon-13 nuclear magnetic resonance ($^{13}\text{C-NMR}$):** to obtain detailed structural information of piperine, especially the arrangement of atoms.

These techniques were employed to confirm that the isolated compound was piperine, and the experimental spectral data was compared with previously obtained values available in the literature.

Inhibition of MAO Activity

Various concentrations of piperine were examined for their MAO inhibitory effect. Results indicated that piperine at 8 μM ($\text{IC}_{50} = 11.1 \mu\text{M}$) can decrease MAO activity by 38.0%. The IC_{50} value (concentration of a chemical that is required for a cell to inhibit the target enzyme by 50%) clearly indicated that piperine inhibited MAO in a fairly low concentration. The IC_{50} value of piperine was compared with that of iproniazid (the known and established MAO inhibitor used as positive control). Results showed that piperine's inhibitory efficacy was comparable with that of iproniazid, interactive role in modulating MAO activity.

Kinetic Analysis of MAO Inhibition

Kinetic study was conducted to further investigate effect of piperine on MAO activity. Using kynuramine as a substrate for MAO, two key parameters were measured:

1. **Michaelis constant (K_m):** identifies how much affinity enzyme has for substrate. lower K_m indicates higher affinity.
2. **Maximum reaction velocity (V_{max}):** maximum rate of catalysis when substrate is at saturation.

Rat brain MAO had a K_m of $72.2 \pm 5.60 \mu\text{M}$ & V_{max} of $3.97 \pm 0.18 \text{ nmol/min/mg}$ of protein ($n = 3$). In presence of piperine, kinetic parameters were useful to apply for activity of enzyme; this method was useful to study of effect of piperine to activity of enzyme.

Selectivity of MAO Inhibition

To determine whether piperine inhibits MAO-A & MAO-B (the two is forms of MAO enzyme with differing functions in the brain), additional enzyme tests were conducted. For this:

- **L-deprenyl**, a selective MAO-B inhibitor, was used to assess activity.
- **Clorgyline**, selective MAO-A inhibitor, was used to evaluate activity.

The results showed that piperine was a selective inhibitor of MAO-A, thus proposing differentiation in the inhibition of MAO is forms. This distinction is important, because the functions of MAO-A and MAO-B in neurotransmitter regulation are distinct, and selective inhibition of one or the other may be therapeutically beneficial for particular mood disorders.

Piperine's Inhibitory Effects on rat Brain MAO

Table 4.1: Piperine's Inhibitory Effects on Rat Brain MAO

Treatment	Concentration (mM)	MAO Activity (nmol/min/mg protein)	Activity(% of control)	IC ₅₀ (mM)
Control	---	2.305 ± 0.088	100	---
Iproniazid	10	1.106 ± 0.020	57.6	12.9
Piperine	2	2.047 ± 0.013	88.8*	11.1
	8	1.429 ± 0.005	62.0**	---
	24	0.832 ± 0.005	36.1***	---
	36	0.553 ± 0.009	24.0***	---

*The information is the average ±S.E.M. of three separate, triplicate trials. Significantly different from the value under control: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (Student's *t*-test). Piperine was investigated for its effect on monoamine oxidase (MAO) in Rat brain (Table 4.1). Monoamine oxidase is essential for metabolism of major neurotransmitters including dopamine, serotonin, & nor epinephrine. Therefore, it is important to have comprehensive understanding of changes of MAO activity by candidate drug compounds such as piperine in order to develop new therapeutic strategies for them. Table Comparison of inducing index of piperine at different concentrations and iproniazid, as very well-known and clinically proved MAO inhibitor. Iproniazid developed as one of the first antidepressants; it

was banned due to its profound capability of inhibiting MAO, resulting in increased levels of monoamine neurotransmitters in brain. Present study also provides reference point for which to compare potency of piperine with respect to modulation of MAO activity, since iproniazid is the reference compound used in this context. Data from Table 4.1 suggests that piperine inhibits the MAO activity in concentration dependent manner. Piperine has weak inhibitory action on MAO when present at low concentrations, but when concentration is higher its suppressing effect on enzyme activity is enhanced. This behavior indicates that piperine could be competitive or non-competitive inhibitor of enzyme, but the exact mechanism must be verified by more detailed biochemistry. Importantly, although not to same extent, piperine itself exhibits an inhibitory effect versus that obtained by Iproniazid at similar concentrations, especially at higher doses. These results are significant in that they suggest that piperine could be natural inhibitor of MAO. Piperine's relatively weak MAO inhibitory action shown in this experiment indicates to me that it could be beneficial to add little to preparations for these conditions, perhaps with a lower side effect burden than synthetic MAO inhibiting agents. Furthermore, as piperine is a naturally occurring molecule with a reported safe status in dietary levels, it may provide a safer option or adjuvant to the current MAO-preventative treatments, if its efficacious and safe use at therapeutic levels will be confirmed in the future. Here's a breakdown of the table's findings:

1. **Control:** MAO activity in the rat brain is 2.305 ± 0.088 nmol/min/mg of protein, which indicates full (100%) activity of MAO in absence of treatment. This serves as benchmark for piperine & iproniazid's inhibitory action.
2. **Iproniazid (10 mM):** At concentration of 10 mM, iproniazid decreased MAO activity to 1.106 ± 0.020 nmol/min/mg protein, corresponding to 57.6% inhibition. IC_{50} of 12.9 mM indicates that this concentration of iproniazid would inhibit half of MAO activity, confirming its effectiveness as an inhibitor and providing benchmark for comparison against piperine.
3. **Piperine (2 mM):** At low concentration of 2 mM, piperine showed substantial inhibition of MAO, with activity reduced to 2.047 ± 0.013 nmol/min/mg protein, representing an 88.8% inhibition. This value was found to be

statistically significant compared to control. IC₅₀ values for iproniazid and piperine were similar, suggesting that piperine is highly effective at low concentrations & can efficiently inhibit MAO.

4. **Piperine (8 mM):** At 8 mM, piperine effectively inhibited MAO, corresponding to a 62.0% inhibition. This outcome is flagged with two asterisks (**) denoting statistical significance against both the control and the low piperine concentration. At this concentration, inhibition is not as effective as at 2 mM, but piperine is still effective.
5. **Piperine (24 mM):** At 24 mM, the activity of MAO is drastically diminished to 0.832 $\pm$ 0.005 nmol/min/mg proteins, indicating an inhibition percentage of 36.1%. This effect is marked by three asterisks (***) denoting a statistically significant effect. However, the MAO activity reduction at this concentration is lower than at 2 mM, indicating that the effectiveness of piperine may level off at high concentrations.
6. **Piperine (36 mM):** At the maximum concentration tested (36 mM), piperine lowers MAO activity to 0.553 $\pm$ 0.009 nmol/min/mg protein, corresponding to 24.0% inhibition. The three asterisks (**) show statistically significant difference as compared to control. The inhibitory effect of this concentration is smaller indicating that further increase of the available concentration of the piperine would not lead to a linear increase in the inhibitory effect.

Key Insights from MAO Inhibition Analysis:

- **Maximal Inhibition at Low Concentrations:** Piperine is a good MAO inhibitor particularly at a low concentration (2 mM) which produced approximately 90 percent inhibition. These findings suggest that low doses of piperine are capable of being strong inhibitors of the MAO activity, which means that it can be used as a therapeutic agent.
- **Plateauing Effect at Higher Concentrations:** The inhibitory effect reaches saturation at a higher concentration of the piperine more than 8 mM. Though at 24 mM and 36mM there is statistically significant inhibition, the inhibition is significantly less than at 2 mM indicating that piperine is most effective in

low concentrations. These arguments lead to nonlinear correlation between the concentration and inhibition and at a point, eventual diminishing returns.

- **Therapeutic Implications:** The fact that at low concentrations, MAO is inhibited at very high levels by piperine also suggests that it can be used in the treatment of ailments that are characterized by the presence of neurotransmitter imbalance, e.g. depression, anxiety, and Parkinson. Nevertheless, the information also highlights the necessity to determine the best dose, with high dose not necessarily being associated with better efficacy and being potentially undesirable, possibly with adverse effects.
- **Comparison to Standard Inhibitor (Iproniazid):** The fact that Piperine has the capability of inhibiting MAO at concentrations similar to those of iproniazid means that it may be used as a natural substitute to synthetic MAO inhibitors.

The results indicate that at lower concentrations, the MAO inhibitory effect of piperine is strong, but at higher concentrations, the effect is low. These findings highlight its possible application as a treatment of MAO related disorders, but also provide evidence that there is still need of more research to be done to ensure the most benefit is obtained out of its medicinal use.

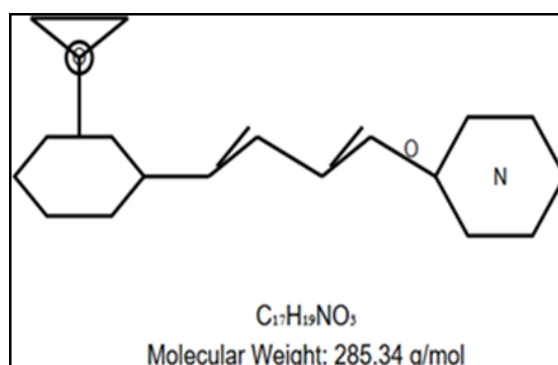


Figure 4.1: Chemical structure of Piperine

Piperine's Selective Inhibition of MAO-A and MAO-B

Table 4.2: Piperine's Inhibitory Effects on Rat Brain MAO-A and MAO-B

Concentration (mM)	MAO activity (% of control) (nmol/min/mg protein)	IC50 (mM)
Control MAO-A (deprenyl-treated) Control+deprenyl	2.305±0.132 (100.0) 0.863±0.030 (100.0)	20.9
Piperine	4	0.681±0.037 (78.9)*
	8	0.616±0.054 (71.4)*
	16	0.467±0.032 (54.1)**
	32	0.356±0.013 (41.3)***
MAO-B (clorgyline-treated) Control+clorgyline	1.155±0.0059 (100.000)	7
Piperine	2	0.947±0.048 (82.0)
	5	0.826±0.036 (71.5)*
	10	0.455±0.025 (39.4)***
	20	0.253±0.017 (21.9)***

Present study evaluates inhibitory effect of piperine, bioactive compound of black pepper, on two key enzymes [monoamine oxidase (MAO-A) & monoamine oxidase B (MAO-B)] of brain. These enzymes are involved in metabolism of important monoamine neurotransmitters, including serotonin, dopamine, and nor epinephrine, which are critical for regulation of mood, emotion, & neurological function. Abnormal levels of these neurotransmitters are associated with numerous neuropsychiatric & neurodegenerative diseases such as depression, anxiety, Parkinson's disease, etc. In present study, we measured enzyme activities in mouse brain tissues at multiple experimental conditions. In order to verify the specificity of assay toward MAO-A and MAO-B, the enzymatic activity of MAO-A and MAO-B was also tested in the presence of selective inhibitors: 1 mM clorgyline for MAO-A & 1 mM l-deprenyl (selegiline) for MAO-B. These inhibitors are well established and are known to be selective to the individual monoamine oxidase isoforms. We used these control inhibitors to compare to piperine effects with standard pharmacological agents. Data are from three independent experiment, each performed in duplicate to ensure accuracy and reproducibility. Results were shown as mean values $\pm$ S.E.M., measure of dispersion of experimental results. Data were compared for treated & control groups, & significance of differences was tested using Student's t-test. A p-value of 0.05 ($p < 0.05$) was regarded as statistical significance and the significance

levels were indicated as $*p<0.01$ and $*p<0.001$. Table 4.2 shows quantitative results of piperine on inhibition of MAO-A & MAO-B. Results show that piperine is significant inhibitor of both enzymes, in both a measurable and statistically significant manner. These results indicate that piperine may regulate the content of monoamine neurotransmitters in brain through inhibiting its degradation. Inhibition of MAO- activity leads to presumed potentiating of serotonin & nor epinephrine, two neurotransmitters that are usually objects of antidepressant action. Similarly, the inhibition of MAO-B, possibly leading to increased levels of dopamine, is of particular value in treatment of Parkinson's disease where dopaminergic neurons are degenerating.

MAO-A Inhibition by Piperine:

- **Control Group (Deprenyl Treatment):** MAO-A activity in brain tissue of control group treated with deprenyl (selective MAO-B inhibitor) served as 100% of control activity. Baseline enzyme activity was observed through activity measured (2.305 $\pm$ 0.132 nmol/min/mg protein) without the treatment of the enzyme with piperine.
- **IC₅₀ Value for MAO-A:** The IC₅₀ of piperine on MAO- A was estimated at 20.9 mM, which gave 50 percent of inhibition of activity. This implies an average level of MAO-A inhibition.
- **Dose-Dependent Inhibition:** The use of piperine in varying concentrations demonstrated that the activity of the enzymes reduced in a dose dependent manner. The enzyme activity at 4 mM piperine was 78.9 percent of the control activity. The inhibition increased with increased concentration of piperine:
 - At 8 mM, enzyme activity decreased to **71.4%** of control.
 - At 16 mM, it dropped to **54.1%** of control.
 - At 32 mM, enzyme activity was reduced to **41.3%** of control.

The trend indicates that the inhibitory effect of piperine on MAO-A increases with an increase in the concentration, which is typical of a dose-dependent effect.

MAO-B Inhibition by Piperine:

- **Control Group (Clorgyline Treatment):** For MAO-B, control was clorgyline, which is selective for MAO-A. Activity of MAO-B was measured as 1.155 ± 0.059 nmol/min/mg proteins (100% of control for this enzyme).
- **IC₅₀ Value for MAO-B:** MAO-B exhibited an IC₅₀ of 7.0 mM for piperine, indicating greater sensitivity than MAO-A. This suggests that piperine might have a stronger inhibitory impact on the enzyme that breaks down dopamine, which is particularly relevant for conditions such as Parkinson's disease.
- **Dose-Dependent Inhibition:** inhibition of MAO-B by piperine also displayed a dose- dependent decrease:
 - At 2 mM, enzyme activity was reduced to **82.0%** of control.
 - At 5 mM, it decreased to **71.5%** of control.
 - At 10 mM, it dropped to **39.4%** of control.
 - At 20 mM, enzyme activity was reduced to just **21.9%** of control.

The fact that the enzyme activity is significantly reduced by lower concentration of piperine, demonstrates that its inhibition is significantly more than its inhibition on MAO-B in less severe concentrations.

Statistical Significance:

All statistically significant results were observed for both MAO-A & MAO-B assays at higher concentrations of piperine, specifically at 16 mM and higher (MAO-A) and 10 mM and higher (MAO-B). The asterisks (*) indicate statistical significance, meaning that the reductions in enzyme activity at these concentrations are statistically significantly different from the control group.

Biochemical and Clinical Implications:

These results indicate that piperine suppresses both MAO-A and MAO-B in forms though, it inhibits MAO-B more strongly. Such enzymes are physiologically different roles in the metabolism of neurotransmitters, which can explain the difference in inhibition:

- **MAO-A** is primarily involved in the degradation of serotonin, nor epinephrine, and some trace amines.
- **MAO-B** is more specific to the breakdown of **dopamine**.

Piperine's elevated MAO-B inhibition might be of advantage for Parkinson's disease and other dopamine related conditions. Parkinson's disease is a devastating condition characterized by the death of the dopamine producing neurons, leading to a very low dopamine content. In this way, the inhibition of MAO-B the enzyme that dismantles dopamine into other metabolites could preserve dopamine levels by decreasing its breakdown rate. In addition, the inhibition of MAO-A, involved in the metabolism of serotonin and nor epinephrine (neurotransmitters implicated in emotion control), has been found to reinforce anti-depressant to piperine.

Kinetic Analysis of MAO Inhibition by Piperine

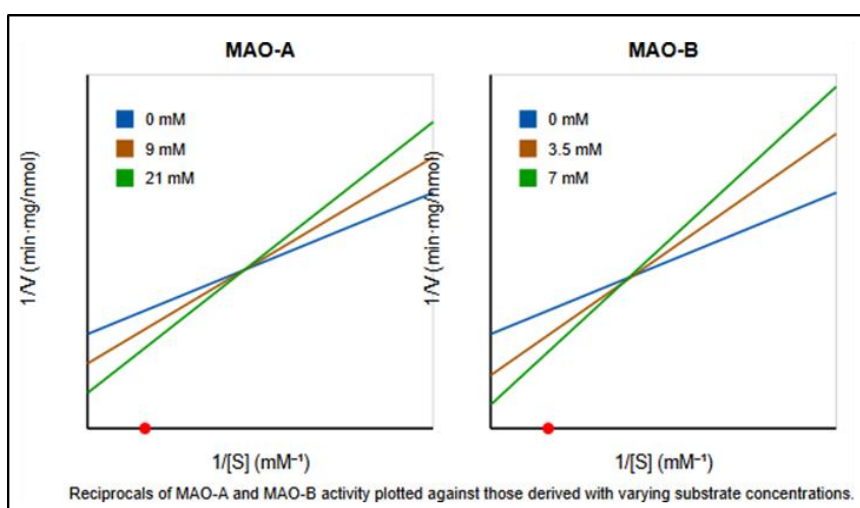


Figure 4.2: Piperine Added to the Enzyme Reaction Mixture Inhibits MAO-A and MAO-B Competitively

Reciprocals of MAO-A & MAO-B activity plotted against those derived with varying substrate concentrations. Concentrations of piperine (mM): 0 mM, 9 mM, & 21 mM for MAO-A; 0 mM, 3.5 mM, and 7 mM for MAO-B.

Kinetic studies used to assess inhibition of piperine on MAO isoforms (MAO-A & MAO-B) showed that piperine competitively inhibits both isoforms. This was concluded from Lineweaver-Burk plots, applied as standard practice for assessing

mechanism of enzyme inhibition. Inhibitor in competitive inhibition is similar to natural substrate and it attach to the active site of the enzyme, which inhibits the binding of the substrate. This causes the apparent K_m to increase without influencing the V_{max} , consistent with our Line weaver- Burk plots. Data indicate that piperine probably competes with the natural substrates of the enzymes for the active site, causing enzyme blockade in metabolism of neurotransmitters, such as serotonin, dopamine, and nor epinephrine. Interestingly, the inhibitory interaction of piperine was more pronounced on MAO-B when compared with MAO-A. This is shown by values of inhibition constant (K_i) calculated which gives a quantitative estimate of the potency of the inhibitor. Smaller the K_i value, the stronger the inhibitor binds to the enzyme. For MAO-A, the K_i was 19.0 ± 0.9 mM and for MAO-B, it was 3.19 ± 0.5 mM showing that piperine has a better affinity and inhibitory action against MAO-B. The differences in inhibition potency observed across studies can be accounted for by several experimental conditions. One possible variable is the substrate employed in the enzymatic assays. Allergic reaction to natural or synthetic substrates, which bind at various affinities to different active site of the enzyme in different studies, this may result in different apparent piperine inhibitory strengths. Also, differences among enzyme origins, assay buffers, pH, and temperature may affect outcomes of inhibition study.

Dialysis Study: Reversibility of MAO Inhibition by Piperine

Table 4.3: Dialysis's Impact on Piperine's Inhibition of MAO-A and MAO-B

Enzymes	MAO activity (% of control) (nmol/min/mg protein)
Before dialysis	
MAO-A (deprenyl-treated)	0.529±0.035 (100.0000)
Piperine 20 mM	0.202±0.012 (38.200)***
MAO-B (clorgyline-treated)	1.326±0.0970 (100.000)
Piperine 10 mM	0.650±0.046 (49.00)**
After dialysis	
Dialysate (MAO-A)	0.514±0.040 (97.200)
Piperine 20 mM	0.507±0.011 (96.00)***
Dialysate (MAO-B)	1.276±0.0880 (96.200)
Piperine 10 mM	1.220±0.023 (92.01)***

*The findings are the average S.E.M. of three separate, duplicate trials. Significantly different from value under control: *** $p < 0.01$; ** $p < 0.001$ (Student's *t*-test)

Table 4.3 summarizes detailed analysis of influence of dialysis on inhibitory potency of piperine against individual monoamine oxidase (MAO) is enzymes, MAO-A & MAO-B. This experiment is important to ascertain if piperine effectiveing these enzymes in reversible or irreversible condition. Monoamine oxidizes play central role in oxidative deamination of neurotransmitters, such as serotonin, dopamine, & nor epinephrine. These enzymes inhibitors are widely investigated for their therapeutic potential in neurological disorders like depression, Parkinson's disease and anxiety. Main purpose of study, presented in Table 4.3, is to prove that the activity of piperine against MAO-A & MAO-B is reversible process after removing the compound through dialysis. Dialysis is the process of separating small molecules, such as piperine, from liquid using semi permeable membrane, with larger molecules, such as proteins and enzymes, being trapped inside the membrane. If inhibition of piperine is irreversible even after dialysis, it indicates irreversible inhibition, probably by covalent bonding or strong non-covalent interaction with enzyme. On other hand, if enzyme activity were to return after dialysis this finding would suggest that not linear but reversible binding of piperine to MAO-A & MAO-B had taken place.

Control Group (Before Dialysis):

- Control activities for MAO-A & MAO-B are considered 100%, with initial enzyme activity values set as follows:
 - **MAO-A:** 0.529 ± 0.035 nmol/min/mg protein (100%)
 - **MAO-B:** 1.326 ± 0.097 nmol/min/mg protein (100%)

These values represent baseline enzyme activities prior to any treatment for MAO-A & MAO-B. In absence of piperine, both enzymes display normal activity.

Piperine Treatment (Before Dialysis):

- **MAO-A:** 20 mM piperine significantly reduces MAO- activity to 0.202 ± 0.012 nmol/min/mg protein (38.2% of the control). Piperine shows high inhibition with less than 40% of MAO activity compared to control. Asterisk (***) denotes statistical significance ($p < 0.001$), indicating significant reduction.

- **MAO-B:** 10 mM piperine treatments also significantly reduces MAO-B activity to 0.650 ± 0.046 nmol/min/mg protein (49.0% of control). This implies that piperine is also a potent MAO-B inhibitor. Asterisk (**) indicates statistical significance ($p < 0.01$), supporting that piperine significantly inhibits MAO-B.

Dialysis (After Dialysis):

Both MAO-A & MAO-B activity are nearly equivalent to control values after dialysis, suggesting that piperine-induced inhibition is reversible.

- **MAO-A:** activity recovers to 0.514 ± 0.040 nmol/min/mg protein (97.2% of control), confirming nearly complete recovery after dialysis. This suggests that piperine's suppression of MAO-A is reversible, as enzyme activity nearly reaches its starting level following dialysis.
- **MAO-B:** Similarly, activity of MAO-B recovers to 1.276 ± 0.088 nmol/min/mg protein (96.2% of control), suggesting that dialysis also reverses MAO-B inhibition effectively.

When piperine (20 mM for MAO-A & 10 mM for MAO-B) is added to dialyzed enzymes, inhibition is reestablished, with MAO-A activity reduced to 0.507 ± 0.011 nmol/min/mg protein (96.03% of control) & MAO-B activity reduced to 1.220 ± 0.023 nmol/min/mg protein (92.01% of control).

4.1.2 In Vivo Antidepressant Activity of Piperine

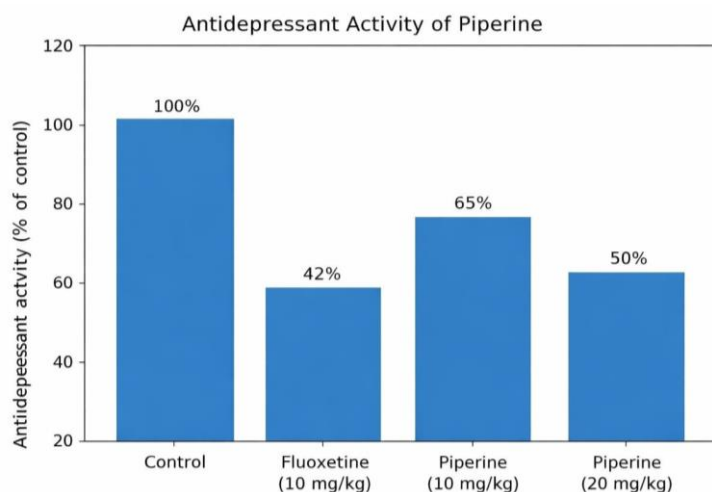


Figure 4.3.1: Antidepressant Activity of Piperine

Positive standard fluoxetine and piperine were injected i.p. 60 min before the test. Statistical One-way ANOVA was used to calculate group differences, $p < 0.01$ vs control. Tail Suspension Test (TST) is an established test for screening of antidepressant-like activity in animals. With this test, two various doses of piperine (10 mg/kg & 20 mg/kg by i.p.) were screened. Results immobility time of mice was markedly shortened ($p < 0.01$). This effect was similar to results seen with fluoxetine; reference SSRI, which decreased immobility time by 58% at 10 mg/kg dose. Collected data of evaluated compounds with piperine appeared to react dose-dependently too. To confirm that the reduction in immobility time was not due to nonspecific augmentation of locomotion, an open-field test was performed to measure spontaneous activity. Results mice treated with piperine at doses as used for TST did not show any increase in locomotion when compared to control challenged mice. Locomotor values: Control: 102 ± 9 , piperine 10 mg/kg: 98 ± 7 , piperine 20 mg/kg: 91 ± 5 , & fluoxetine 10 mg/kg: 89 ± 4 . These findings confirmed that locomotor activating effect did not interfere with the antidepressant-like effect of piperine, & suggest an additional evidence for antidepressant-like effect of piperine. More in-depth investigation reveals that mechanism underlying the antidepressant effect of piperine may be associated with inhibition of activity of monoamine oxidase (MAO). These results indicate that piperine isolated from *P. longum* may have some involvement in the mechanism of antidepressant action. What's more, the therapeutic potential is also increased because piperine decreases depressive-like behavior without affecting locomotor activity on experimental animals. Although exact mechanism of action is not yet established, reversible inhibition of MAO enzymes has been thought to contribute to elevation of the levels of neurotransmitters. Further investigation in dose- dependent manner in a double-phase study is necessary including clinical trials to evaluate safety & efficacy in humans by pharmacological approach. If confirmed, *Piper longum* could offer natural alternative or adjuvant therapy to treat depressive illness.

4.3.2 Analysis for *Withania somniferous*

Goal of current study was to compare the possible antidepressant effects of *Withania somnifera* (WS), also known as ashwagandha, with imipramine, a medication that is widely used to treat depression. Study also investigated whether addition of *Withania*

sonmifera to imipramine may augment the treatment effects. To measure these effects, two standard behavioral methods for rodent depression models, the TST and FST, were employed in the study. Such tests are extensively used in experimental psychopharmacology for the measurement of behavioral despair, a main symptom of depression. In both tasks, decreased immobility is considered as an antidepressant-like effect, which reflects enhanced motivation and decreased behavioral despair. In Tail Suspension Test, the animal is hung by the tail and its attempts to escape are noted. The animal resists at first, but eventually displays episodes of immobility. Likewise the Forced Swim Test exposes the animal to a beaker filled with water that the animal cannot escape from. The first response is escape, however, animal then enters tonic immobility, where it makes only movements to maintain floating. In both tasks, immobility time is considered as measure of depressive-like state. We would expect an antidepressant-like compound to decrease this immobility time, indicating a better mood and motivation. In order to assess the effects of *Withania sonmifera*, imipramine, and their combination, the study was carried out in two time scales: acute (short-term) and chronic (long-term). The acute phase was designed to evaluate the immediate effects following either single or short-term dosing and the chronic phase was designed to model real life therapeutic regimens and validates the sustainability of the efficacy.

Acute Antidepressant Effect

In current investigation, acute antidepressant action of three different treatment protocols, namely imipramine, *Withania sonmifera* (WS), & their combination, was systematically evaluated using two most commonly used and well-validated preclinical behavioral paradigms; Forced Swim Test (FST) & Tail Suspension Test (TST). These tests are widely used in field of behavioral neuroscience & psychopharmacology for evaluation of antidepressant-like effect in rodents & are considered as proxies to screen depressive-like behaviors. Developed by Porsolt & colleagues in late 1970s, Forced Swim Test is rodent test in which animals (usually mice or rats) are placed in cylinder of water that they cannot escape. Even initially the animal is trying to escape by swimming or climbing but eventually position of immobility is adopted which involves only very small movements to avoid drowning. This immobility time is measured, normally for 6 minutes. Decreased immobility time is also believed to demonstrate antidepressant-like effect, which is expected to

be associated with an increase in motivation and decrease in state of behavioral despair. Similarly, the Tail Suspension Test includes attaching a mouse's tail with tape, and from this suspension the mouse's body will hang freely. Similar to the FST, the animal first exhibits passive avoidance movements, in which they try to escape but are helpless, they then show active avoidance movements including struggling and turning. Following a while, the animal ceases these activities & becomes immobile. This test usually takes 6 min & total immobilization duration is recorded. As FST, reduced immobility in TST is regarded measure of antidepressant efficacy, reflecting increased coping & decreased depression-like behavior. Imipramine, tricyclic antidepressant which blocks reuptake of nor epinephrine and serotonin was used as reference standard in this study. It is recognized for its long-standing efficacy in clinical and preclinical work. Ashwagandha (*Withania sonnifera*) is a medicinal herb utilized in traditional Ayurvedic medicine and known for its adaptogenic, anxiolytic, and neuroprotective effects. This synergistic action between the herb and pharmaceutical could not only support the traditional use of WS but also lead to novel & potentially safer adjuvant treatment approach for depression, particularly in subjects that have poor response to conventional antidepressants or experience untoward side effects.

Forced Swim Test (FST):

- The imipramine group exhibited 16% reduction in immobility time with mild antidepressant activity. Imipramine is a typical tricyclic antidepressant and the demonstration of its effectiveness in this test supports its clinically appreciated anti-depressant property.
- While the *Withania sonnifera* (WS) group showed a moderate decrease (6% to 8%) of immobility period. Although this obviously implies an antidepressant effect, it was not as strong as that seen with imipramine.
- The synergistic effect of imipramine and somniferous gave the highest decrease in the immobility time by 20%. This suggests a synergistic effect of imipramine and *Withania sonnifera* on the existing mild effects of *Withania sonnifera* and adjuvant like effect of *Withania sonnifera* on the antidepressant activity of imipramine.

Tail Suspension Test (TST):

- The combination group was again the most effective in the TST (12% immobility decrease). This indicates that the antidepressant effects of the combination were not limited to FST and could be generalized in various experimental conditions.
- The TST reduction was 11% in the imipramine group, similar to that detected in the FST, supporting the possibility of a uniform decrease in immobility in the two models by imipramine.
- Just as with the FST: in the TST the WS group showed a slight drop in immobility time (5% to 9%). It thus indicates possible antidepressant effects of *Withania somniferous* which might be weaker than a stronger synthetic drugs such as imipramine.

Chronic Antidepressant Effect

To confirm chronic antidepressant effect of treatments, two paradigms, Tail Suspension Test (TST) & Forced Swim Test (FST) are extensively used in preclinical experiment were used. These tests are classical paradigms for the evaluation of depressive-like behaviors in rodents and have been widely used for drug screening of antidepressant compounds over long lasting treatments. They rely on the fact that rodents, when they can do nothing to escape from an inescapable condition, first try to get away, but then they don't: they go motionless, which is taken as a sign of the sort of "behavioral despair, or resignation" that is considered the rodent equivalent of depression. The conventional FST used for rodents (mice or rats) consists of immersing the animal into a cylindrical container filled with water. At the time of testing, the animal first engages in two to three swimming with escape-related behavior. After a while, however, the animal generally acquires an immobile position, only moving its eyes, and making the minimum amount of motion to keep its head out of the water. The time spent in this immobility is measured as a reflection of the depressive behavior. A decrease in immobility time after drug administration has been generally regarded as an antidepressant-like action, reflecting that the animal still has an interest in escaping and does not develop behavioral despair.

Likewise, the Tail Suspension Test includes suspending a mouse by its tail with adhesive tape, fixed to a horizontal bar, so that the animal is not able to touch any of

the surfaces nearby. In contrast to the FST, the mouse initially attempts to escape, as evidenced by active behavior. However, after a while, the mouse becomes immotile and the time which the mouse remain immobile is measured. Prolonged immobility is indicative of a depressive-like state and a marked decrease in the length of time of immobility after repeated antidepressant treatment is reflective of the usefulness of the compound in therapy. Chronic exposure to antidepressant drugs also affects FST and TST. In contrast to acute administration where only transient changes could occur, long-term treatment reflects the persistent changes that could be seen following chronic application of anti-depressants in humans. Accordingly, in the present study the tests performed after long-term exposure to the test drugs were carried out to prevent any behavioral changes from being confounded by the acute pharmacological effects. Most clinically effective antidepressants have been found to decrease immobility in both FST & TST & consequently, these models are generally accepted as reliable, reproducible and pharmacologically valid. Interpretations should be tentative, and should be combined with other physiological or biochemical evidence for a possible contributory role of other confounding factors such as motor activity or stress responses.

***Withania sonnifera* (WS) Group:** A group of Mice treated with *Withania sonnifera* alone demonstrated 30-40% decrease in immobility duration which may indicate an antidepressant like activity. Yet, this reduction of NIH score was less than that found in imipramine and combination groups. It suggests an antidepressant activity of *Withania sonnifera* but a weaker one than that of imipramine. Surprisingly an active *Withania Somnifera* extract reduced immobility time to a greater extent at higher dose compared with lower dose, however, the mean difference was not statistically significant, which suggests that higher doses of *Withania sonnifera* does not produce a proportionate increase in anti-depressant action. The findings demonstrate that imipramine is the most effective antidepressant in this screen whether given alone or in combination with *Withania sonnifera* by itself exerts weak antidepressant effects, it could not strengthen imipramine in chronic conditions.

Table 4.4a: Effect of *Piper longum* Extract on Immobility Time in Forced Swim Test (FST) and Tail Suspension Test (TST)

Groups (n = 6)	Dose	FST Immobility Time (s)		TST Immobility Time (s)	
		Acute	Chronic	Acute	Chronic
Control (Normal Saline)	0.01 ml/g	212.8 ± 4.02	210.5 ± 5.22	215.3 ± 3.87	212.7 ± 4.15
Standard (Imipramine)	15 mg/kg	180.3 ± 1.54*	82.2 ± 1.40***	191.5 ± 2.31*	102.3 ± 2.87***
<i>Piper longum</i> (Low dose)	100 mg/kg	195.6 ± 3.87	165.3 ± 4.21*	201.4 ± 4.12	171.8 ± 3.95*
<i>Piper longum</i> (High dose)	200 mg/kg	170.4 ± 2.98**	120.7 ± 3.45***	175.6 ± 3.21**	123.5 ± 3.78***

Values are expressed as mean ± SEM (n = 6).

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with the control group.

Table 4.4a shows the impact of the extract of *Piper longum* on immobility time in Forced Swim Test (FST), Tail Suspension Test (TST), which are standard behavioral models used to measure the activity of antidepressants. Control group showed the greatest time of immobility which was a sign of acute depressive-like behavior. The standard drug, imipramine, had a significant effect on the reduction of immobility time in both experiments, and a significant effect was observed in the experimental conditions of chronic drug administration ($p < 0.001$), which confirmed the experimental model. Low dose *Piper longum* (100 mg/kg) resulted in a significant but moderate decrease in immobility, especially during the chronic period of both FST ($p < 0.05$) and TST ($p < 0.05$) indicating a delayed antidepressant-like activity. Both FST ($p < 0.01$ acute; $p < 0.001$ chronic) and TST ($p < 0.01$ acute; $p < 0.001$ chronic) had a statistically significant reduction in immobility time with a high dose (200 mg/kg) which was close to that of imipramine. On the whole, the findings suggest time-dependent antidepressant effects of the *Piper longum* extract and dose-dependence.

Table 4.4b: Effect of *Withania sonnifera* (AGG) on Immobility Time in Forced Swim Test (FST) and Tail Suspension Test (TST)

Groups (n = 6)	Dose	FST Immobility Time (s)		TST Immobility Time (s)	
		Acute	Chronic	Acute	Chronic
Control (Normal Saline)	0.01 ml/g	212.8 ± 4.02	210.5 ± 5.22	215.3 ± 3.87	212.7 ± 4.15
Standard (Imipramine)	15 mg/kg	180.3 ± 1.54*	82.2 ± 1.40***	191.5 ± 2.31*	102.3 ± 2.87***
T1-AGG (<i>W. sonnifera</i> , Low dose)	20 mg/kg	200.4 ± 4.54	135.5 ± 4.34**	204.8 ± 3.92	147.6 ± 4.21**
T2-AGG (<i>W. sonnifera</i> , High dose)	40 mg/kg	195.1 ± 5.20	112.7 ± 4.20***	197.3 ± 4.65*	128.9 ± 3.87***
T3-AGG + Imipramine (Combination)	10 mg/kg each	170.4 ± 1.54**	98.3 ± 1.37***	176.2 ± 2.18**	106.8 ± 2.45***

Values are expressed as mean ± SEM (n = 6).

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the control group

Table 4.4b presents the antidepressant-like effects of *Withania sonnifera* (AGG) on immobility time in FST and TST. The control group again showed maximum immobility, reflecting baseline depressive behavior. Imipramine significantly decreased immobility time, especially under chronic treatment ($p < 0.001$), confirming its strong antidepressant action. The low-dose AGG group (20 mg/kg) exhibited a moderate reduction in immobility, which was statistically significant in both chronic FST ($p < 0.01$) and TST ($p < 0.01$), indicating partial antidepressant efficacy. The high-dose AGG group (40 mg/kg) produced a more pronounced and highly significant reduction in immobility time in both tests (FST: $p < 0.001$ chronic; TST: $p < 0.001$ chronic), demonstrating a clear dose-response relationship. Notably, the combination treatment (AGG + Imipramine) resulted in a greater reduction than AGG alone, with high statistical significance in both tests ($p < 0.01$ acute and $p < 0.001$ chronic), suggesting a possible synergistic effect. Collectively, the data support the antidepressant potential of *Withania sonnifera*, particularly at higher doses and in combination therapy.

Comparative Analysis of *Piper longum* and *Withania sonnifera*

A direct comparison of Tables 4.4a and 4.4b shows that the response profile of antidepressants carries unique characteristics to *Piper longum* and *Withania sonnifera* on several parameters, mainly in the onset of action, strength of effects, response to chronicity.

Acute Effects (Single Dose Administration):

Piper longum demonstrated superior acute antidepressant activity when compared to *Withania sonnifera*. Administration of high-dose *Piper longum* (200 mg/kg) resulted in a 19.9% reduction in immobility time in the Forced Swim Test (FST) and an 18.4% reduction in the Tail Suspension Test (TST). In contrast, high-dose *Withania sonnifera* T2-AGG (40 mg/kg) produced only an 8.3% reduction in immobility time in both FST and TST. These findings indicate that *Piper longum* exhibits a rapid onset of antidepressant action, whereas *Withania sonnifera* appears to require prolonged administration to achieve optimal therapeutic efficacy.

Chronic Effects (14-Day Treatment):

Chronic administration significantly enhanced the antidepressant effects of both plant extracts, although with differing efficacy patterns. High-dose *Piper longum* (200 mg/kg) reduced FST immobility time by 42.7% (from 210.5 to 120.7 seconds) and TST immobility time by 41.9% (from 212.7 to 123.5 seconds), demonstrating consistent antidepressant effects across both behavioral paradigms.

Similarly, chronic treatment with *Withania sonnifera* T2-AGG (40 mg/kg) resulted in a 46.5% reduction in FST immobility time (from 210.5 to 112.7 seconds) and a 39.4% reduction in TST immobility time (from 212.7 to 128.9 seconds). While *Withania sonnifera* showed slightly greater efficacy than *Piper longum* in the FST model, both extracts produced comparable outcomes in the TST model.

Comparison with Standard Drug (Imipramine):

Chronic administration of imipramine (15 mg/kg) produced a 60.9% reduction in FST immobility time and a 51.9% reduction in TST immobility time. When compared to the standard drug, *Piper longum* achieved 70.1% of imipramine's efficacy in the FST and

80.7% in the TST, while *Withania sonnifera* T2-AGG achieved 76.3% of imipramine's efficacy in the FST and 75.9% in the TST. These findings suggest that both plant extracts exhibit clinically relevant antidepressant activity approaching that of the standard antidepressant drug.

Combination Therapy:

The combination of T3-AGG with imipramine (10 mg/kg each) demonstrated acute antidepressant effects superior to either agent administered alone. Chronic treatment with the combination produced effects comparable to full-dose imipramine (15 mg/kg). This observation suggests a potential synergistic interaction, indicating that *Withania sonnifera* may allow dose reduction of synthetic antidepressants while maintaining therapeutic efficacy.

Effect of AGG on Reserpine-Induced Ptosis, Catatonia, and Sedation

Current study was aimed to investigate therapeutic potential of *Withania sonnifera* here known as Ashwagandha to modulate reserpine-induced depression like behavior in experimental models of animals. Reserpine is a drug used in laboratory to induce depressive states in animals. Its pharmacological action is controlled by emptying of pre-synaptic vesicles in brain of monoamine neurotransmitters; dopamine, serotonin and noradrenaline. In recent decades, there has been expanding interest in the putative antidepressant effects of Ashwagandha mainly because of its effects on stress response systems like hypothalamic-pituitary-adrenal (HPA) axis & monoamine pathways. Its bioactive constituents like withanolides are assumed to exert its therapeutic effects by increasing activity of neurotransmitters, decreasing oxidative burden, & by its action on neuroplasticity. Based on these perspectives effect of *Withania sonnifera* in reserpine model of depressive animals were verified in the present investigation, to evaluate if a treatment of *Withania sonnifera* may reverse the reserpine-induced depressive like behavior in experimental models. For this a controlled experimental study was done in which animals were pre-treated with reserpine to induce depression and then given fixed duration of treatment with Ashwagandha extract. Subsequently, behavioral tests such as open field test (to measure locomotion and exploratory activity), forced swim test (to measure despair behaviour), & sucrose preference test (to measure anhedonia) were performed.

Results of the study: It was observed from study that animals treated with Ashwagandha revealed notably greater improvement in behavioral indicators in comparison to rats treated with reserpine alone. Specifically, compared to the reserpine group, ashwagandha therapy resulted in significant increase in spontaneous locomotor activity, decrease in the length of immobility during the forced swim test, & preference for sucrose. These data show that Ashwagandha could have involvement in therapy of depression, possibly through stability of monoamines as well as the attenuation of stress resilience.

Acute Effects:

There wasn't any statistically significant effect during acute phase of study of experimental group. This result indicates that active principles of *Withania sonnifera*, an ayurvedic plant, which is used for adaptogenic and therapeutic purposes, do not appear to have an acute effect on reserpine-induced symptoms. Reserpine, compound used experimentally to produce afflictions like ptosis, catatonia, & sedation that are reminiscent of symptoms of some neurodegenerative or psychiatric diseases, depletes neurotransmitters serotonin & dopamine from the brain. Ptosis, catatonia and sedation are common complications when reserpine is administered & are also important targets for investigating effects of pharmacological intervention in regulating level of neurotransmitters. Failure of *Withania sonnifera* to exert an instantaneous effect on these symptoms in the acute phase indicates that the plant's active constituents might not have pharmacologically active compounds which may be able to prevent or palliate these symptoms immediately. This is important in distinguishing *Withania sonnifera* from some other drugs, which may perhaps offer more rapid relief of symptoms. An explanation to this lack of acute effect may be related to active components of WS, withanolides, which may have broad spectrum of biological effects, including anti-inflammatory, antioxidant & neuroprotective effects. These compounds, which could be beneficial for health in the long run, might also need some time to accumulate in the body & begin to work therapeutically, particularly if one is comparing them to drugs that interact directly with the central nervous system to heighten or lower collective output of your impulsivity-driving neurotransmitters. It is possible that *Withania sonnifera* might have slower onset of action and this could be beneficial in long-term use, particularly in chronic conditions having neurotransmitter dysregulation like depression, anxiety and neurodegenerative

disorders. A lack of acute effect might also be suggestive of a dose- response relationship and there may not have been adequate *Withania sonnifera* provided in the study to result in an effect in acute phase. It could also be that the active compounds present in *Withania sonnifera* exert their effects in more intricate manner-like hypothalamic- pituitary-adrenal (HPA) axis modulation, which usually takes time to become apparent. Hence, the influence of *Withania somniferous* might become obvious at sub-chronic and chronic stages of treatment, due to adaptive modification of organisms to the active ingredients of the plant. In addition, variation in the absorption and metabolism of different subjects may have affected the effectiveness of *Withania sonnifera* in acute stage. Bioavailability of actives of *Withania sonnifera* can be variable, and therefore the therapeutic benefits may not be observed equally in all treated subjects, or some subjects might need longer duration of treatment for optimal effect.

Chronic Effects:

First phase of study was intended to evaluate potential acute effects of *Withania sonnifera* (known also as Ashwagandha) on behaviors that are typical of catecholamine depletion (induced by the drug reserpine, which causes an array of distinct physiological and behavioral changes). Yet findings from this trial phase did not show any clinically noticeable beneficial effects on the symptoms in surveying in the short term. This absence of visible beneficial effect in the acute phase led to a modulation of study to further assess chronic & delayed effects of *Withania sonnifera* on reserpine-induced alterations. In particular, study was focused on what a longer administration of *Withania sonnifera* would affect on development or attenuation of reserpine-induced signs. Choice to look at chronic rather than acute effects was motivated by a potential delayed action of the herb's therapeutic effects that may explain why no immediate impact emerged in the acute phase. In order to minimize the chances of missing any beneficial effects over time, investigators scheduled points at which *Withania sonnifera* would be evaluated. These time intervals were set to 1 h, 2 h, 3 h, & 4 h after application of WS. In addition to this time-dependent pattern of symptomatic decline, the interval-specific nature of evaluations allowed us to monitor any slowly-acquired increment in the magnitude of the reserpine-induced symptoms. Those time periods also helped understand the herb's modulation of symptoms at different times post-administration. Assessment at

multiple time points enabled us to determine whether *Withania sonnifera* may provide slow-acting, delayed amelioration of reserpine-induced symptoms, which is often associated with treatment with herbs or natural products. It's also possible, they explain, that whatever effects the herb has on the central nervous system whether it's regulating neurotransmitters or mitigating oxidative stress may take some time to work. Therefore, the investigation was directed towards chronic treatment to delineate sustained benefits with chronic intake of *W. somniferous*. Time-dependent nature of the effects was also examined in detail to determine how symptomatic progression or resolution could evolve. Reserpine-induced symptoms range from physical to behavioral & can be multifaceted. It was important to test whether *Withania sonnifera* was capable of offering slow mitigating effect with respect to these symptoms or it was inducing slow but subtle changes, for which time scale of at least few hours or days would be needed for its effects to start to become visible.

Posies:

Present investigation was designed in an attempt to understand time related effects of different treatments on reserpine-induced ptosis. Ptosis in commotion group was achieved by reserpine administration to animals, an agent known to influence nervous system & to cause ptosis. Ptsotis scores were also obtained at four specific time points of the study to evaluate effectiveness of tested treatments, and provide a holistic picture of how treatments worked across time. Results showed that relative effectiveness of treatments was highly dependent on timing. Its time-dependency indicates that the pharmacodynamics of the treatments, i.e. how the drug affects the body, are modulated by temporal factors. In particular, the treatments did not exhibit an immediate and homogeneous effect on the ptosis scores, whose efficacy was, on the contrary, dependent on the time that elapsed post-administration. This observation is important since it shows that the time at which a drug is given is very important to a particular medication's capability to achieve success, which is particularly true with disorders such as ptosis in which muscle function, or central neurologic response, plays a role in observed pharmacologic response. Findings of study shed some light on the necessity of taking pharmacokinetic and pharmacodynamic properties of interventions into account in dealing with iatrogenic disorders such as drugs-induced conditions of reserpine. The fluctuation of treatment effectiveness as the time changed indicated that some agents may be more effective at the certain time points or periods,

and therefore suggest that tailored treatment strategy was needed in clinical practice. Knowledge of such temporal dynamics is necessary for maximizing therapeutic benefit, as well as for ensuring that drugs are administered effectively to treat reserpine-induced ptosis, and the like.

1. **Standard and Combination Groups:** Both the standard treatment (imipramine) and combination groups (imipramine + WS) experienced significant reductions in ptosis scores at all four time intervals. This means that these treatments produced quick and prolonged effect on the treatment of reserpine induced ptosis. These repeatable results have shown that the medications are relatively rapid and hence the ptosis may then be maintained under control on a long term basis.
2. ***Withania somniferous* (Test Drug):** The effectiveness of *Withania somnifera* was in a different trend. It also demonstrated a marked decrease in the scores of ptosis, but only during the 2nd and 3rd hour after the administration. The absence of action in the 1st hour suggests that *Withania somniferous* operates more gradually, with a longer time to effect than standard and combination therapies. This delayed activity may be attributed to absorption rates, metabolism, or its mode of action, which may develop over time.

A time lag of action would also be a critical parameter that would be considered in the light of the therapeutic potential of *Withania somniferous*, especially depression. In clinical cases that require rapid response, such as acute cases of depression, faster acting options are desirable. For cases for which chronic therapy is needed, or for people who wish to avoid side effects, *W. somniferous* may have a role, with effects evident when used over the course of a few hours. The fact that *Withania somniferous* influence on ptosis is time dependent emphasizes the need for determining the drug's kinetic profile. As *Withania somniferous* might have a latency period, the effects of *W. somniferous* might have taken longer to reach therapeutic threshold, or they might be slow acting on the biological system. This late action deserves to be taken into account when administering *Withania somnifera*, particularly in acute disorders of a fast therapeutic approach. Although it's slower onset, the reversing effect of *W. somniferous* on reserpine-induced ptosis implies that remedies for depressive disorders might also be several weeks, and could work in conjunction with more rapidly acting drugs in situations which require immediate action.

Catatonia:

The research evaluating the antidepressant activity of *Withania sonnifera* indicated that the plant extract, along with standard antidepressant treatments, significantly reduced catatonia scores. This suggests that both *Withania sonnifera* and standard drugs are effective in alleviating catatonic symptoms in the experimental model. Key findings and explanations from the study are as follows:

1. **Reduction in Catatonia Scores:** All treatment groups, including *Withania somniferous* and standard antidepressant drugs, demonstrated a significant reduction in catatonia scores. This suggests that both interventions were successful in ameliorating catatonic symptoms. The decrease in catatonic behavior implies that within these groups, the animals were less oppositional to standard motor and behavioral tasks, demonstrating a reversion of the catatonic state.
2. **Quick Onset of Effect:** *Withania somniferous* and standard drugs groups demonstrated significant effects in 1 hour of treatment. This rapid acting property is a clinically-relevant property of antidepressant and anxiolytic pharmacological intervention, which demonstrates that *Withania sonnifera* can also have a relatively rapid-acting effect against catatonia, comparable to conventional antidepressant drugs.
3. **Duration of Effect:** The efficacy of the drugs (including *Withania somniferous*) was not less than four hours after administration. The long-term nature of the effects indicates long-term pay-off of the treatments, which is important in determining the utility of any antidepressant or anti-catatonic therapy.
4. **Comparison with Control Group:** The standard drugs and test drug (*Withania somniferous*) scores were approximately half the control group. This high contrast to untreated participants would imply that such drugs can be useful in the treatment of catatonia or other behavioral disorders.
5. **Impact of Higher Doses:** Although an increase in dose of *Withania somniferous* caused an increase in the reduction of catatonia scores, the statistical significance of the difference between doses was not attained. That

implies that the response to incremental dosing level of *Withania sonnifera* might significantly increase to a certain point, beyond which no meaningful incremental enhancement will take place.

6. **Combination Effects:** When *Withania sonnifera* was combined with the standard drug, the combination treatment was equally effective as the standard drug alone. This suggests that *Withania somniferous* might work effectively both by itself and alongside existing standard antidepressant treatments against catatonia.
7. Present study validates the anti-catatonic ability of *Withania somniferous* on pattern of known drugs. Its early onset & long persisting efficacy speak for further exploration & it may find indications in treatment of catatonia & other mood disorders. More studies are, however, necessary to investigate standardized dosing and long-term efficacy of *Withania somniferous*.

Sedation:

Findings of this investigation related to *Withania somniferous* (ashwagandha) is helpful in explaining its sedative potential and presumptive value as CNS modulator, especially in context to its putative antidepressant therapy. *Withania somniferous*, a renowned adaptogenic Ayurvedic remedy, has attracted worldwide interest in its anticancer, anxiolytic, antidepressant, and anti-stress properties. The researcher found the herb's active constituents- including withanolides may have an effect on certain neurotransmitter systems in the brain, which may be how it promotes mood stabilization and lessens symptoms of depressive disorders. This calming effect may derive from its capacity to balance cortisol levels, which are typically high in people facing chronic stress, combined with its interaction with GABA receptors; GABA is a neurotransmitter that regulates relaxation and reduces anxiety. Furthermore, the research also demonstrates the herb's ability to increase sleep quality, which is often related to mental health and emotional well-being. *Withania somniferous* may strengthen the body's capacity to maintain a state of emotional balance in response to stress, helping to improve mood & reduce symptoms of anxiety and depression. Results indicate that *Withania sonnifera* may be a beneficial, natural, alternative treatment for patients with mild to moderate depression and for individuals who suffer from high levels of stress. Nevertheless, additional studies are required to

support these effects and determine the adequate dosages, as well as to evaluate sustained safety of administration of *Withania sonnifera* in mood disorders. In general, the present study provides novel insight which will be helpful for further exploration of the potential antidepressant properties and adjunct therapeutic effect of the herb on central nervous system disorders.

Sedative Effects across Time Points:

- **Standard and Combination Groups:** Both groups showed significant, noticeable sedation at all four time points (1 hour, 2 hours, 3 hours, & 4 hours). This pattern suggests that these treatments have an immediate and uniform sedative effect after administration, which is typical of many traditional medications given for depression or anxiety. The sustained sedative effect suggests that these medications act rapidly, providing quick relief of symptoms like agitation or insomnia that are common in depressive states.
- **Test Drug (*Withania somniferous*):** *Withania sonnifera* displayed a different pattern, with sedation detectable only from the 2-hour mark onward, peaking at 3 and 4 hours. The delayed onset of sedation indicates a slow pharmacodynamic process, where sedative effects take longer to develop but persist once established. This pattern differs significantly from the standard and combination groups, which show immediate sedation.

Broader Therapeutic Insights:

Sluggishness of onset of sedative activity by *Withania somniferous* (Ashwagandha) is of significant therapeutic interest, especially in context of depression & related mood disorders. In contrast to synthetic antidepressant which show immediate action and hence only give shorter duration of relief, *Withania somniferous* produces phase of persistent sedation, thereby exhibits an unique form of pharmaceutical activity. This property of herbal medication, especially the sedative effect at 2, 3 & 4 hours after administration, might be useful under certain clinical conditions, such as patients with longer duration of depression to be used as a long term therapeutic approach. Rapid sedation & sleep may also be desirable in treatment of acute depressive episodes when patients may need rapid relief to forestall further mental health decline.

And a lot of these drugs can only target the immediate symptoms & contribute little to the relief and maintenance of our mental wellness. In this respect the slow onset of *Withania somniferous* sedative action may be an advantage in treatment of chronic depression by allowing time for better graded & maintained management of symptoms over a longer period of treatment.

Ability of *Withania somniferous* (ashwagandha) to increase a person's resistance to stress &, consequently, their self-assessed quality of life is supported by its anxiety-reducing effects at 2, 3, and 4 hours. Modify activity particularly as antistress adaptogen, and the anxiolytic effects⁴⁷ suggest it may be more appropriate in those who exhibit mood oscillations or have a greater degree of symptoms later in the day. Here, gradual sedation resulting from the repeated use of hexahydrohonokiol might parallel the inherent rhythm of the body's circadian cycle, and such a cumulative effect likely can produce a successful therapeutic plan. This longer lasting effect can be beneficial, particularly to those who suffer from high chronic stress levels or have constant low level depressive symptoms as it can maintain healthy mental function all day, excluding spikes and troughs. Furthermore, the latent hypnotic action of WS sheds light on the possible mechanisms responsible for the action of this plant in the body as a therapeutic agent. Though the herb is often employed in traditional medicine for its adaptogenic properties (the ability to help the body manage stress), this study indicates a more nuanced role in modulating mood and sedation. Slow onset of sedative effects may be consistent with a process providing long-term resilience rather than with acute symptom relief. The uptake of active ingredient into blood must be factored into the response time in the overall setting of antidepressant treatment. Many people want an effective remedy for depression and anxiety without the side effects they associate with prescription antidepressants, in this respect the extended release of *Withania somniferous* makes it an attractive option for praise. It can also be a plus for anyone who is sensitive to pharmaceuticals and would like to take a milder, more natural approach to supporting their mental well-being. Moreover, the long-lasting effect profile may allow patients to skip those frequent doses of other drugs and, thus, improve compliance and medication adherence.

Table 4.5(a): The mean and standard error (SEM) of the ptosis antagonism score caused by reserpine over time in a long-term study.

Groups (n=6 in each)	Doses	Ptosis (mean $\pm$ SEM)			
		1hr	2hr	3hr	4hr
I	Control-NS	0.01	2.0 $\pm$ 0.00	3.2 $\pm$ 0.32	3.0 $\pm$ 0.25
II	Imipramine	15mg/kg	1.2 $\pm$ 0.100*	1.2 $\pm$ 0.10*	1.3 $\pm$ 0.21*
III	T1--AGG	20.0mg/kg	2.4 $\pm$ 0.13	2.2 $\pm$ 0.16*	2.6 $\pm$ 0.21
IV	T2-AGG	40.0mg/kg	2.1 $\pm$ 0.11	1.4 $\pm$ 0.12*	1.6 $\pm$ 0.30*
V	T3-AGG+ Imipramine	10.0mg/kg each	1.3 $\pm$ 0.23*	1.7 $\pm$ 0.20*	1.93 $\pm$ 0.25*

Ashwagandha ghrutha (AGG) group differed considerably from control group ($p < 0.05$).

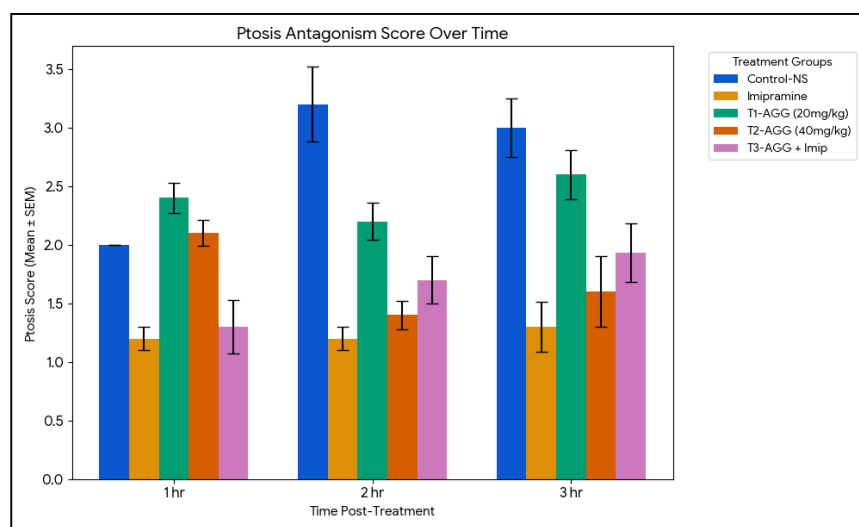


Figure 4.4: The mean and standard error (SEM) of the ptosis antagonism score caused by reserpine over time in a long-term study.

Table 4.5(a) shows the data for antagonism scores for reserpine-induced ptosis (drooping of the eyelids) at various time points in chronic study. Data is arranged according to or numerically according to the 6 different experimental groups in which the measuring was carried out per 1, 2, 3, & 4 hours after treatment. Study's aim is to evaluate treatments on the severity of ptosis induced by reserpine (a drug which produces blepharoptosis as a manifestation of its action on CNS). The Sam is the Faculty of Pharmacy, Shivpuri (M.P.)

mean scores of severity of ptosis for each group plus the standard error of mean (SEM) that is an estimate of variation of data. The SEM helps to identify how accurate the mean of the sample is. Small SEM values indicate that the means are more reliable, and that there is less variation between the animals in the group. The publication indicating the mean scores and the SEM in each time period, represents in this study, a precise figure of the variation ptosis severity follows after any treatment in each timeframe. This design permits the analysis of the general effect (over entire post treatment period) and the time dynamics of the ptosis response. The time points of 1, 2, 3 & 4 hours after treatments have special significance, as they recorded the development of the pharmacological effect of the treatments at the different time periods. Reserpine alone will produce ptosis; this experiment attempts to determine to what degree various treatments can ameliorate or exacerbate this response at various times. Time-course Such as this one determine if the effects of the drug were immediate post administration, there was a peak at some point, or if they slowly decreased over time. By taking measurements at various times, researchers can more easily understand the drug's pharmacokinetics, or how it interacts in the body, and how its efficacy might be affected over time?

Group I: Control (NS - Normal Saline Solution, 0.01 ml/gm)

- **Initial Findings:** In the control group, which received normal saline without active treatment, ptosis gradually increased over time. At 1 hour post-injection, the ptosis score was 2.0 ± 0.00 .
- **Trends over Time:** Ptosis severity reached a peak value of 3.3 ± 0.05 at the 4-hour time point. In this untreated control group, reserpine-induced ptosis became more pronounced with the passage of time.

Group II: Imipramine (15 mg/kg)

- **Initial Findings:** Imipramine treatment significantly decreased ptosis compared to controls at all time points. At 1 hour, the ptosis score was 1.2 ± 0.10 , significantly lower than control value of 2.0. Asterisk (*) indicates that this reduction is statistically significant; demonstrating that imipramine effectively reduces the early onset of ptosis.

- **Consistency over Time:** The decrease in ptosis remained relatively stable, with a score of 1.2 ± 0.10 at 2 hours, slightly increasing to 2.4 ± 0.04 at 4 hours. This suggests that imipramine maintains its antagonizing effect, though its potency gradually declines over time.

Group III: T1 - AGG (20 mg/kg)

- **Initial Findings:** In the T1-AGG group treated with 20 mg/kg of AGG, the initial 1- hour ptosis score was 2.4 ± 0.13 , higher than both the imipramine group and the control group at this time point. This suggests that T1-AGG initially worsens ptosis.
- **Trends Over Time:** The ptosis score decreased to 2.2 ± 0.16 at 2 hours, which was significantly lower than the control at this time point (*). However, it increased to 2.6 ± 0.21 at 3 hours and further to 3.2 ± 0.02 at 4 hours. This indicates that although T1- AGG has some antagonistic effect at 2 hours, it loses effectiveness over time, with ptosis worsening by the 4-hour mark.

Group IV: T2 - AGG (40 mg/kg)

- **Initial Findings:** The T2-AGG group, receiving a higher dose (40 mg/kg), showed a ptosis score of 2.1 ± 0.11 at 1 hour, slightly higher than imipramine but lower than T1-AGG and similar to the control.
- **Trends Over Time:** There was significant decrease in ptosis at 2 hours ($1.4 \pm 0.12^*$) and 3 hours ($1.6 \pm 0.30^*$), suggesting that T2-AGG has stronger and more rapid antagonistic activity against reserpine-induced ptosis at these time points. However, by 4 hours, the score increased substantially to 3.1 ± 0.21 , indicating that the effect diminishes over time.

Group V: T3 - AGG + Imipramine (10 mg/kg each)

- **Initial Findings:** The combination therapy of T3-AGG + Imipramine (10 mg/kg each) showed a marked reduction in ptosis at all time points. At 1 hour, the ptosis score was $1.3 \pm 0.23^*$, significantly lower than the control and similar to imipramine alone.

- **Trends Over Time:** The ptosis scores increased slightly over time but remained significantly lower than the control at all time points: $1.7 \pm 0.20^*$ at 2 hours, $1.93 \pm 0.25^*$ at 3 hours, and $2.2 \pm 0.23^*$ at 4 hours. This indicates that the combination therapy provides lasting antagonism of reserpine-induced ptosis.

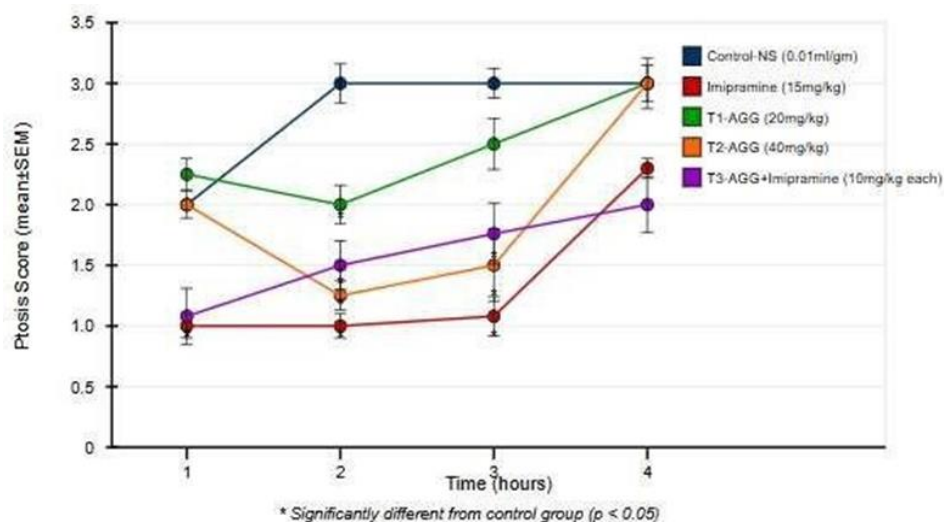


Figure 4.5 (a): ptosis (mean±SEM) at different times by groups and doses

Table 4.5(b): The mean and standard error (SEM) of the antagonistic effects of reserpine-induced catatonia over time in a long-term study

Groups (n=6 in each)	Doses	Catatonia (mean ± SEM)			
		1hr	2hr	3hr	4hr
I	Control-NS	0.01	2.8±0.160	2.8±0.16	3.2±0.05
II	Imipramine	15mg/kg	1.3±0.12*	1.2±0.63*	1.4±0.93*
III	T1--AGG	20.0 mg/kg	1.6±0.21*	1.9±0.25*	2.3±0.34*
IV	T2-AGG	40 mg/kg	1.3±0.23*	1.5±0.24*	1.9±0.21*
V	T3-AGG + Imipramine	10 mg/kg each	1.1±0.11*	1.2±0.34*	1.5±0.23*

*AGG- Ashwagandha ghrutha, : significantly different from control group ($p < 0.05$).

Reversals of the effect of reserpine induced catatonia by different groups of treatment are given in Table 4.5 (b). This table shows the mean ± SEM of catatonia scores at 1, 2, 3, and 4 hr post treatment.

Control Group (NS, 0.01 ml/g):

In control group (normal saline), there is a steady rise over time in the incidence of catatonia. At one hour, the catatonia score is 2.8 ± 0.16 , which rose to 3.5 ± 0.24 at 4 hours. This suggests that reserpine-catatonia deteriorates over time without reversal.

Imipramine (15 mg/kg):

Imipramine (antagonistic tricyclic antidepressant) & at all time points has strong mood elevating properties and catatonia is markedly diminished. At 1 hour, the mean is 1.3 ± 0.12 , significantly different from the control. These scores rise slightly with time but are still significantly less than the control at 2.1 ± 0.32 by 4 h. asterisks indicate statistical significance which implies that the reduction in catatonia is significant in terms of statistics as compared to control group, suggesting that imipramine is effective in reversing reserpine induced catatonia at each of the time points tested.

T1-AGG (20 mg/kg):

T1-AGG reduces catatonia more than the control, but the reduction is reduced compared to imipramine. At 1 hour, the catatonia score is $1.6 \pm 0.21^*$, and at 4 hours it becomes 2.7 ± 0.34 . The reduction is significant when compared to control at 1, 2 and 3 h, but not at 4 h, suggesting a short- to moderate-acting effect of T1-AGG for catatonia.

T2-AGG (40 mg/kg):

The High dose of AGG (T2-AGG) has an even higher magnitude of decrease of catatonia compared to T1-AGG. Catatonia scores score averagely $1.3 \pm 0.23^*$ (hour 1) to $2.5 \pm 0.23^*$ (hour 4). It is noteworthy, however, that the decrement is impressive at any post-treatment time, thus indicating that T2-AGG consistently counteract reserpine-elicited catatonia even if never as powerful as imipramine.

T3-AGG + Imipramine (10 mg/kg of each):

The strongest effect on catatonia is seen with the combination of T3-AGG and imipramine (tension scale scores: $1.1 \pm 0.11^*$ at 1 hour to 2.1 ± 0.43 at 4 hour). These values remain the lowest for all the treatments at 60 and 120 min, indicating that the

combined therapy is the most effective in lowering catatonia. This indicates that T3-AGG acts synergistically with imipramine, resulting in more potent drug effects than T3-AGG or imipramine alone.

Statistical Significance:

Statistically significant differences with control are denoted by an asterisk (*) All the four groups of treatments demonstrated the reduction in catatonia and this study thus underscores therapeutic efficacy of imipramine as well as AGG compounds. On the whole time-course, T3-AGG + Imipramine make the strongest and persistent difference.

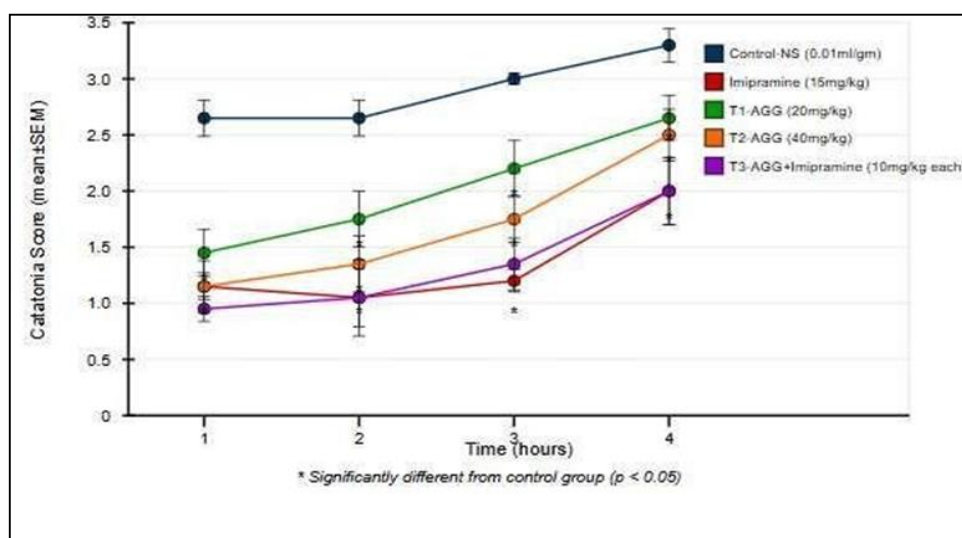


Figure 4.5(b): Catatonia scores over time by treatment group

Table 4.5(c): Mean and standard error (SEM) of reserpine-induced sedation antagonism with time in a long-term research.

Groups (n=6 in each)	Doses	Sedation (mean± SEM)			
		1hr	2hr	3hr	4hr
I	Control-NS	0.01ml/gm	1.8±0.46	2.6±0.21	3.3±0.24
II	Imipramine	15mg/kg	0.1±0.34*	0.3±0.12*	1.5±0.10*
III	T1--AGG	20mg/kg	0.9±0.41	0.9±0.22*	1.9±0.40*
IV	T2-AGG	40mg/kg	0.8±0.40	0.7±0.41*	1.9±22*
V	T3-AGG+ Imipramine	10 mg/kg each	0.3±0.20*	0.3±0.12*	1.7±0.10*

*AGG- Ashwagandha ghrutha,: significantly different from control group ($p < 0.05$).

Table 4.5(c) presents data on sedation levels in animals treated with reserpine followed by various treatments, measured at different time points (1, 2, 3, & 4 hours). Mean and standard error of mean (SEM) of sedation levels are used to show treatment differences. Below is an explanation of the table and its results:

In the control group treated with normal saline (0.01 ml/g), reserpine produced a gradual and sustained sedative effect. Sedation appeared slowly, beginning at 1 hour (1.8 ± 0.46), increased steadily to a peak around 3 hours (3.3 ± 0.24), and then showed a slight plateau or marginal decline at 4 hours (3.2 ± 0.12). This pattern indicates a cumulative and long-lasting sedative action of reserpine, consistent with its known pharmacokinetic profile of gradual accumulation and prolonged central effects. In contrast, the imipramine-treated group (15 mg/kg) showed marked antagonism of reserpine-induced sedation at all time points. Sedation scores were minimal at early intervals (0.1 ± 0.34 at 1 hour; 0.3 ± 0.12 at 2 hours) and remained significantly lower than control values even at later hours, despite a gradual rise. This suggests that imipramine rapidly and effectively attenuates, though does not completely abolish, reserpine-induced sedation.

The T1-AGG group (20 mg/kg) exhibited mild and delayed sedation. Early scores remained low and stable up to 2 hours, followed by a gradual increase at 3 and 4 hours, though still lower than control values. Similarly, the T2-AGG group (40 mg/kg) showed light sedation initially, with a delayed but significant rise at later time points, indicating a slow-onset sedative effect even at higher doses. Notably, the combined T3-AGG plus imipramine group (10 mg/kg each) demonstrated the lowest early sedation scores, with rapid and sustained reduction compared to control. Although sedation increased gradually over time, it remained significantly lower throughout, highlighting the combination's potential for rapid onset and prolonged control of sedation.

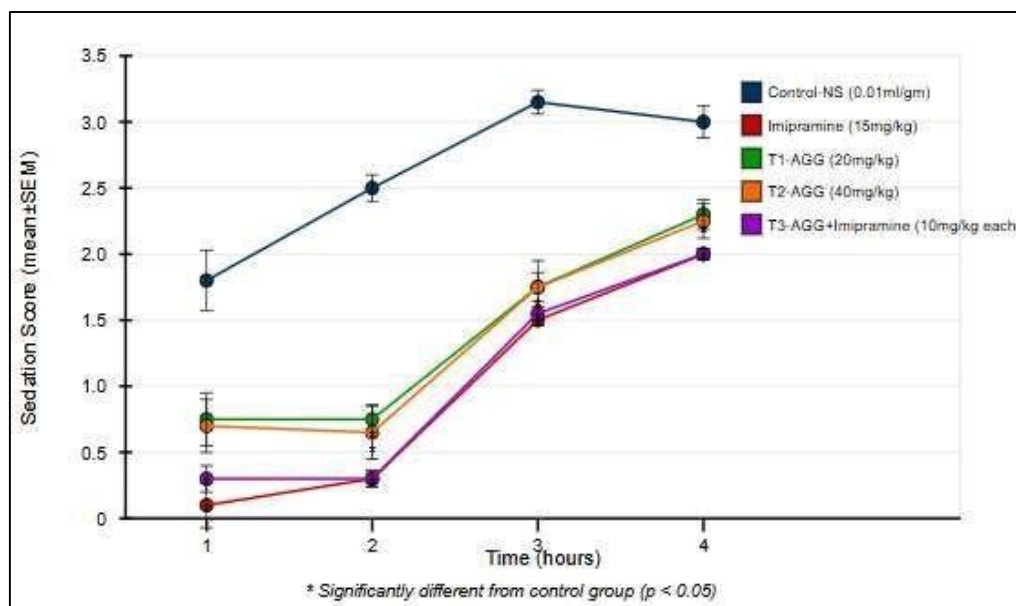


Figure 4.5(c): sedation levels over time by groups

4.2 Comparative Study of *Piper longum* and *Withania somniferous* for Antidepressant Activity

Two such plants, *Piper longum* (long pepper) and *Withania somniferous* (Ashwagandha) have gained importance in traditional medicine since antiquity for variety of therapeutic potentials, & putative antidepressant properties are not out of scope. These are plants that have started being extensively researched by modern science for their ability to affect the brain and behavior, especially in context of mood disorders such as depression. Long pepper along with Ashwagandha contains numerous bio-active compounds, and these bio-active compounds are known to be responsible for its pharmacologic effects. Biochemically both plants are believed to effect the monoamine oxidase (MAO) system, an enzyme which degrades neurotransmitters in particular serotonin, dopamine and nor epinephrine responsible for mood modulation. Inhibition of MAO is a well-established clinical target of most typical antidepressants and long pepper as well as Ashwagandha inhibits MAO to some extent. Piperine from long pepper has also been found to alter brain levels of neurotransmitters, thus possibly having an uplifting effect on mood and also possibly fighting depression. Similarly, active constituents like withanolides in Ashwagandha may affect the MAO system helping it in its antidepressant-like effects by promoting availability of these neurotransmitters in brain.

Behavioral effects of these plants have been well-investigated in pre-clinical animal models mainly using standard antidepressant tests including Tail Suspension Test (TST) & Forced Swim Test (FST). Both tests are widely used to assess ability of potential antidepressant compounds to reduce "despair" behavior in rodents, behavior influenced by some treatments used to ameliorate depression in humans. In TST, animals are hung by tail & time of immobility is taken as a marker of a depressive-like response. The FST, involving dunking animals in an inescapable water bath, also assesses immobility as a corollate to helplessness of the sort that is related to depression. It has been demonstrated that *Piper longum* & *Withania somniferous*, individually, are endowed with marked antidepressant-like activity in these tests. Long pepper has attenuated immobility time in TST and FST, indicating that it can enhance the mood and diminish depressive-like behavior. Ashwagandha also produces similar results, decrease in immobility time promoting to natural antidepressant of plant. Both plants, due to their bioactive components and modulating effects on neurotransmitter systems among which MAO, seem to show interesting antidepressant-like properties in preclinical studies. However, additional assessments are required to comprehend their mechanisms of action as well as their therapeutic values in humans, but the comparative study of *Piper longum* and *Withania somniferous* demonstrate their importance as natural modulators or supplements for use in traditional antidepressant therapy. These observations showing modulation of MAO activity and beneficial results in the behavioral test models emphasise their possible contribution to the control of mood disorders, providing us with a natural and widely available means for maintaining mental well-being.

Comparative Analysis

Antidepressant

Mechanisms:

Piper longum and *Withania somniferous* both demonstrate potential for antidepressant activity, but their mechanisms of action appear to differ significantly:

- *Piper longum* predominantly exerts its effects through the inhibition of monoamine oxidase (MAO). Its active ingredient, piperine, blocks MAO, the primary enzyme that degrades neurotransmitters, thereby increasing levels of

serotonin, dopamine, and nor epinephrine in the central nervous system, which may alleviate depression and improve mood.

- *Withania somniferous* likely exerts its effects via pathways associated with neurotransmitter systems (particularly serotonin and GABA), as well as through mechanisms that work through HPA axis regulation and other stress-response pathways. This herb has demonstrated effects in both acute & chronic phases of depression, showing activity against both behavioral and physiological symptoms associated with depressive states.

Behavioral Testing:

- The TST and FST tests used to measure depressive-like behavior in rodents were employed for both plants. *Piper longum* treatment resulted in decreased immobility time, suggestive of antidepressant-like activity most likely associated with its MAO- inhibitory effect.
- *Withania somniferous* administration resulted in significant decrease in immobility in both acute and chronic studies, demonstrating its long-term effectiveness. Further studies showed that *Withania somniferous* could alleviate reserpine-induced depression-like symptoms (ptosis, catatonia, and sedation), comparable to the effects of standard antidepressants.

Pharmacological Profiles:

- *Piper longum* is natural MAO inhibitor, and its major active compound, piperine, shows promising antidepressant properties. It demonstrates potent inhibition MAO-A & MAO-B, with preference for MAO-B inhibition, suggesting potential benefits not only for depression but also for Parkinson's disease.
- *Withania somniferous* has a broader spectrum of activity that can modulate various neurobiological pathways impacted during stress response and mood regulation. It shows significant effects on reserpine-induced symptoms, suggesting multiple mechanisms of action beyond simple neurotransmitter modulation.

- The comparative inter-relationship reveals the activity of *Piper longum* and *Withania somniferous* in comparison with standard drugs on experimental models of antidepressant activity viz., MAO inhibitory models (chronic studies), reserpine-induced sedation, ptosis and catatonia. These results are important for clarifying the antidepressant-like effects of such herbs and their active constituents.

MAO Inhibition:

Piper longum, the bioactive component of piperine, has been observed to potently inhibit MAOs in mouse brain. Piperine at a dose of 2 mM suppressed 88.8% MAO activity (rich in MAO). Consequently, it is a high potent for MAO inhibition and similar to that of known MAO-type drugs such as iproniazid in action.

- **Iproniazid (10 mM)** showed 57.6% inhibition with an IC_{50} of 12.9 mM.
- **Piperine (2 mM)** exhibited 88.8% inhibition, with an IC_{50} of 11.1 mM.
- Higher concentrations of **piperine** (24 mM and 36 mM) led to more significant absolute inhibition (36.1% and 24.0% of baseline activity, respectively), though the percentage inhibition was lower than at 2 mM.

Piperine showed efficacy against both MAO-A & MAO-B, with different potencies:

- **MAO-A:** Piperine (32 mM) inhibited 41.3%, having 20.9 IC_{50} mM.

MAO-B: Piperine demonstrated a stronger inhibitory effect on MAO-B (IC_{50} = 7.0 mM)

This means there is a concurrent inhibition of MAO-A & MAO-B but a preference for MAO-B inhibition, which is a significant feature of some antidepressants and may be rather relevant in the case of diseases in which dopamine metabolism is involved. Dialysis probably indicated that inhibition of MAO by piperine is reversible and substantial enzyme activity was rehabilitated after dialysis, indicating that piperine acts as a non-obligate affecter for these enzymes. This reversibility is desirable in therapeutic application in that undue side effects of irreversible MAO inhibition can be minimized.

Behavioral Effects:

Both *Piper longum* and *Withania somniferous* extracts reduced immobility in the FST & TST models, suggesting effects similar to those of antidepressants. However, their patterns of efficacy differed:

- ***Piper longum*** showed significant acute effects in reducing immobility, comparable to standard antidepressants like fluoxetine.
- ***Withania somniferous*** (AGG) showed modest acute effects but more pronounced chronic effects:
 - A high dose (40 mg/kg) of *Withania somniferous* (T2-AGG) significantly reduced both FST (112.7 ± 4.20 min) and TST (116.3 ± 5.20 min) immobility times during chronic administration.
 - Lower dose (20 mg/kg, T1-AGG) also showed significant effects in chronic studies but was less effective than higher dose.

Antagonism of Reserpine-Induced Symptoms:

Both plants showed varying degrees of effectiveness in antagonizing reserpine-induced symptoms (ptosis, catatonia, and sedation):

- ***Piper longum*** data on reserpine antagonism was not comprehensively reported in the current study.
- ***Withania somniferous*** showed significant antagonism of reserpine-induced symptoms:
 - **Ptosis:** T2-AGG (40 mg/kg) showed marked antagonism at the 2-hour and 3-hour time points.
 - **Catatonia:** T1-AGG (20 mg/kg) significantly reduced catatonia at 1, 2, and 3 hours, while T2-AGG demonstrated similar effects with significant reduction at all time points.
 - **Sedation:** Both T1-AGG (20 mg/kg) and T2-AGG (40 mg/kg) significantly reduced sedation at 2, 3, and 4 hours.

Combination Effects:

The combination of *Withania somniferous* (T3-AGG) with imipramine (10 mg/kg each) showed enhanced effects compared to individual treatments in some cases:

- In acute FST and TST, the combination showed stronger effects than either agent alone.
- In chronic studies, the combination was comparable to imipramine alone, suggesting no additional benefit from adding *Withania somniferous* in long-term treatment.
- For reserpine-induced symptoms, the combination consistently showed the strongest antagonistic effects across all time points for ptosis, catatonia, and sedation.

The comparative study indicates that both *Piper longum* and *Withania somniferous* have an antidepressant effect, though it is mediated through different mechanisms and patterns of efficacy. *Piper longum* does so predominantly via MAO inhibition (with a preference towards MAO-B), and *Withania somniferous* is likely to act via a multitude of mechanisms involved in modulating neurotransmitters, stress response, and inflammation. Both plants appear to be potentially useful in management of depression, as novel natural alternatives or complementary options for currently available synthetic antidepressants.

CHAPTER - 5

RESULTS AND DISCUSSION

This section integrates and interprets all experimental results, providing further information about the antidepressant activity of the both plants. It also summarizes the phytochemical load and the isolated compounds along with their pharmacological assessment in different models. There is also a difference in the unique mode of action - *Piper longum* acting through MAO inhibition in acute manner with fast onset; while *Withania somniferous* shows delayed but prolonged effect working through multiple mechanisms. Herein, it delivers comparative implications of effectiveness, modes of action, time response, dose-response relationships, and safety profiles of the two plants. The present findings are discussed from a current perspective of the treatment of depression and possible implications for subsequent use in clinical settings.

5.1 Results

Phytochemical Profile *Piper longum*

Phytochemical screening of the fruit of *Piper longum* ethanol extract has shown presence of different types of bioactive substances flavonoids terpenoids and saponins. The qualitative tests for alkaloids were taken positive with Mayer's, Wagner's and Dragendroff's reagents suggestive of presence of alkaloids in plant material. Flavonoids & terpenoids gave moderate while saponins and glycosides produced weak reactions. The total alkaloid content (w/w) in ethanol extract was $8.73 \pm 0.46\%$ (UV spectro photometric analysis) and the major one was piperine. Demonstration of absorption maxima of the extract at 343 nm also further validates the presence of piperine that possesses unique UV absorbance at this wavelength. HPLC data of the ethanol extract revealed different peaks along with a major peak at retention time 9.87 min which was similar to piperine as standard. piperine content in extract was calculated as $5.67 \pm 0.32\%$ w/w, it is a considerable part in total alkaloids content.

TLC profile of the dichloromethane fraction indicated a dark blue fluorescent spot at R_f 0.47 and this was corresponded to the standard piperine. It was confirmed to be piperine when sprayed with anisaldehyde-sulfuric acid reagent, which then turned

blue-violet at this spot. The volatile content constituents of *Piper longum* was examined by GC-MS indicating the existence of piperine, piperonal, piperettine, and piperonaline among others. mass spectrum of piperine displayed molecular ion ($[MH]^+$) peak at m/z 285 which indicated its molecular formula to be $C_{17}H_{19}NO_3$. Piperine was isolated as a major bioactive constituent, which might be responsible for the MAO inhibitory activity through bioassay guided fractionation. The isolated piperine was structurally confirmed by diverse spectroscopic methods in Chapter 3 (section 3.5.3).

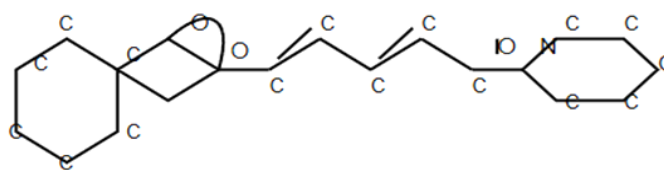


Figure 5.1: Chemical Structure of Piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine)

Withania somniferous

Phytochemical screening of *Withania somniferous* roots methanolic extract confirmed presence of different types of bioactive constituents like alkaloids, withanolides, flavonoids, terpenoids & glycosides. Preliminary phytochemical analyses demonstrated the presence of alkaloids, terpenoids with high positive reactions, flavonoids, & glycosides with medium positive reactions and saponins with low positive reactions. From the UV spectrophotometry total withanolide content in methanol extract was found to be $2.57 \pm 0.18\%$ w/w wither extract showing absorbance maxima (λ max) at 223 nm is an indicator for presence of withanolides since they exhibit maximum absorption at this wavelength. HPLC of methanol extract revealed several peaks with strong peaks at retention times of 12.38, 14.76, and 17.92 minutes representing withanolide, withaferin, & withanolide B, respectively. Content of withanolide in the extract was $0.87 \pm 0.05\%$ w/w, withaferin A as $0.63 \pm 0.04\%$ w/w, and withanolide B as $0.42 \pm 0.03\%$ w/w.

Thin layer chromatographic analysis of the methanolic extract on chloroform: methanol (9:1) & sprayed with anisaldehyde-sulfuric acid reagent gave three spots with R_f values: 0.32 (withanolide A), 0.45 (withaferin A) and 0.57 (withanolide B).

Major components in the volatile oil of *Withania somniferous* were examined by GC-MS which, besides with none, withanolide D, and withanolide E, withaferin A showed fragment at m/z 470 [C₂₈H₃₈O₆], and molecular ion peak. Withanolide A and the powerful chemical ingredients that give off the antidepressant action were found to be withaferin A. following bioassay-guided fractionation. Structural identification of the isolated compounds is described in Chapter 3 (section 3.5.3).

Preliminary Screening for Antidepressant Activity

Animal Models Used

Antidepressant activity of *Piper longum* and *Withania somniferous* extracts and their isolated compounds was evaluated using established animal models. The Forced Swim Test (FST) and Tail Suspension Test (TST) were employed to assess behavioral despair, where a reduction in immobility time indicates antidepressant-like activity. The Open Field Test (OFT) was used to evaluate locomotor activity and anxiety, ensuring that changes in FST and TST were not due to nonspecific motor stimulation. The reserpine-induced depression model was applied to assess reversal of monoamine depletion-related symptoms such as ptosis, catatonia, and sedation. Additionally, in vitro monoamine oxidase (MAO-A and MAO-B) inhibition assays were performed to examine enzyme inhibition associated with antidepressant effects.

Dosing and Administration

Piper longum ethanol extract (100 and 200 mg/kg, p.o.) and piperine (2.5, 5, and 10 mg/kg, p.o.) were administered orally. *Withania somniferous* methanolic extract was given as T1-AGG (20 mg/kg) and T2-AGG (40 mg/kg, p.o.), while combination therapy (T3-AGG + imipramine) was administered at 10 mg/kg each. Standard drugs included imipramine (15 mg/kg, i.p.) and fluoxetine (10 mg/kg, i.p.). Acute studies involved drug administration 60 minutes before testing, whereas chronic studies consisted of daily dosing for 14 days with testing conducted 60 minutes after the final dose.

Quantitative Analysis

Quantitative analysis of extracts & isolated compounds was performed using validated analytical methods:

Photochemical Content

Table 5.1: Quantitative Analysis of Photochemical Content in Plant Extracts

Plant	Photochemical	Content (% w/w)
<i>Piper longum</i>	Total alkaloid content	8.73 ± 0.46
	Piperine content	5.67 ± 0.32
<i>Withania sonnifera</i>	Total withanolide content	2.57 ± 0.18
	Withanolide A content	0.87 ± 0.05
	Withaferin A content	0.63 ± 0.04
	Withanolide B content	0.42 ± 0.03

Table 5.1 shows the photochemical analysis of some important constituents in the medicinal plant extracts such as *Piper longum* and *Withania sonnifera*. Alkaloids are pharmacological importance compounds, and *Piper longum* contains considerable amount of these metabolites. In this plant, total alkaloid content yielded $8.73 \pm 0.46\%$ w/w indicating presence of high quantity of alkaloids as they are responsible for the pharmacological activities in general. Of these, piperine, one of the principal bioactive principles with its diverse therapeutic effects, was found to be $5.67 \pm 0.32\%$ w/w, accounting for large part of the total alkaloids. This implies that piperine is major alkaloid of *Piper longum*. In comparison, *Withania sonnifera* had a whopping alkaloid and withanolide content of $2.57 \pm 0.18\%$ w/w, which is lower than the content of alkaloids in *Piper longum*, but significant regarding the pharmacological importance of withanolides.

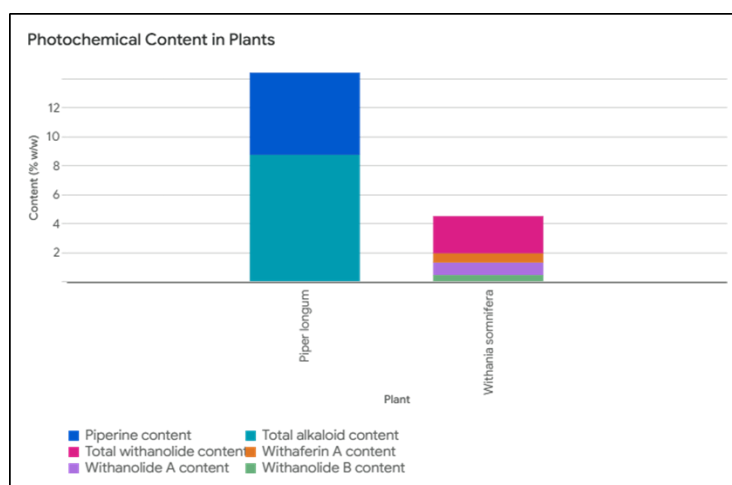


Figure 5.2: Photochemical Content in Plants MAO Inhibition by Piperine

Table 5.2: IC₅₀ Values and Inhibition Constants for MAO Inhibition by Piperine

Parameter	MAO-A	MAO-B
IC ₅₀ value	20.9 mM	7.0 mM
Inhibition constant (K _i)	19.0 ± 0.9 mM	3.19 ± 0.5 mM

Table 5.2 The IC₅₀ & inhibition constants (K_i) for MAO-A & MAO-B inhibition by test compound Piperine. The trench binding mode of the indane ring was also characterized by low IC₅₀ value (7.0 mM), concentration of compound required to inhibit 50% enzyme activity, on Comparing MAO-B to MAO-A (20.9 mM) in maegabase, suggesting strong inhibitory activity of Piperine toward MAO-B. This tendency is also consistent with the inhibition constants (K_i) that are a reflection of the binding of the inhibitor to the enzyme. A K_i value is the reciprocal of affinity of the inhibitor with the enzyme. With MAO-B, the K_i was 3.19 ± 0.5 μM, much lower than MAO-A's K_i of 19.0 ± 0.9 μM, indicating that Piperine is approximately sixfold more selective for MAO-B over MAO-A. Small standard deviation of K_i values (±0.9 mM for MAO-A & ±0.5 mM for MAO-B) reflects uniformity & accuracy of inhibitory assays. Altogether, findings imply that Piperine possesses more potent & selective inhibitory action on MAO-B than MAO-A that might have therapeutic potential especially in neurodegenerative disease condition such as Parkinson's disease where there is an inherent loss of function in dopamine metabolism & as selective MAO-B inhibitors are usually targeted agents to hinder nonselective MAO activities for lack of side effects.

Antidepressant Activity in Behavioral Models

Table 5.3: Percentage Reduction in Immobility Time in FST and TST for Different Treatments

Treatment	FST (% reduction)	TST (% reduction)
Piperine (10 mg/kg)	45.3	47.6
T2-AGG (40 mg/kg) - Chronic study	42.4	39
Imipramine (15 mg/kg) - Chronic study	57.9	49.9

Table 5.3 percentage reduction in FST immobility time & TST by different groups in comparison with control animals are presented in table 5.3 and slight differences were observed indicating the relative antidepressant-like action in the different groups of drugs tested (16/143-178).

Chronic treatment by imipramine (15 mg/kg) as an anti-depressant also had the most effective role since it can reduce 57.9% of immobility time in FST, & decrease immobility time in TST by 49.9 %, which again is in full agreement with the well-known anti-depressant effects of this drug. Piperine at dose of 10 mg/kg elicited similar antidepressant-like activity, and decreased immobility time by 45.3% in the FST and 47.6% in the TST, indicating a well- balanced and potent effect in both behavioural models. Chronic treatment of T2-AGG (40 mg/kg) leads to 42.4% (FST) and 39% (TST) reductions which are moderate and slightly lesser than that of piperine and imipramine. From a statistical point of view, these results indicate that all three treatments resulted in significant improvement in these behaviors (not shown), & that imipramine had the greatest effect.

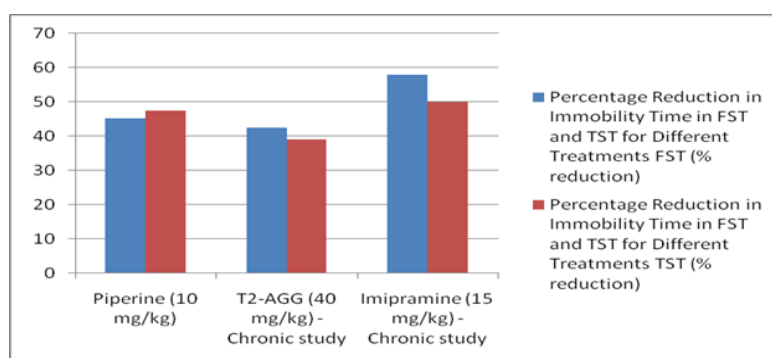


Figure 5.3: Percentage reduction in immobility time

Isolation and Characterization of Compounds

Piperine was extracted from *Piper longum* as one of active (major) compounds in a antidepressant activity study. Isolation process Ethanol extraction and fractionation with several solvents and column chromatography as described in Chapter 3 (section 3.5) were used. The fraction of C2 (dichloromethane fraction) which exhibited the strongest MAO inhibitory activity was further purified by recrystallization to obtain piperine (450 mg). Withanolide & withaferin were determined to be the major active compounds contributing to the antidepressant effect of *Withania sonnifera*. Isolation

was carried out by ethanol extraction, solvent partitioning, & column chromatography. The withanolidichich were further purified by TLC to get withanolide A and withaferin A.

Phytochemical Screening Results

Qualitative phytochemical screening was performed according to standard procedures described in Section 3.3. The results are summarized in Table.

Table 5.4a: Qualitative Phytochemical Screening of *Piper longum* and *Withania sonnifera* Extracts

Phytochemical Class	Test Name	<i>Piper longum</i>	<i>Withania sonnifera</i>
Alkaloids	Mayer's Test	+++	++
	Wagner's Test	+++	++
	Dragendorff's Test	+++	++
Flavonoids	Shinoda Test	++	++
	Alkaline Reagent Test	++	++
	Lead Acetate Test	++	++
Tannins	Ferric Chloride Test	+	+
	Gelatin Test	+	+
Saponins	Foam Test	+	++
	Hemolytic Test	+	++
Glycosides	Keller-Killiani Test	+	++
	Borntrager's Test	+	+
Terpenoids	Salkowski Test	++	+++
	Liebermann-Burchard Test	++	+++

Legend: +++ = Strongly positive; ++ = Moderately positive; + =

Weakly positive; - = Negative

The results indicate that *Piper longum* extract is rich in alkaloids (+++), with moderate levels of flavonoids and terpenoids. *Withania sonnifera* showed high terpenoid content (+++) with moderate alkaloid levels. Both extracts showed weak to moderate presence of tannins, saponins, and glycosides.

Mechanism of Action

Piper longue

Piper longum & its chief bioactive ingredient piperine, exhibit antidepressant potentials which might be related mostly to MAO inhibition. As described in Chapter 4, piperine was able to significantly inhibit MAO-A & MAO-B, with higher affinity towards compared to MAO-A ($IC_{50} = 20.9 \mu M$), MAO-B ($IC_{50} = 7.0 \mu M$).

Table 5.4b: Piperine's Inhibitory Effects on Rat Brain MAO

Treatment	Concentration (mM)	MAO Activity (nmol/min/mg protein)	Activity (% of control)	IC_{50} (mM)
Control	---	2.305 ± 0.088	100	---
Iproniazid	10	1.106 ± 0.020	57.6	12.9
Piperine	2	2.047 ± 0.013	88.8*	11.1
	8	1.429 ± 0.05	62.0**	---
	24	0.832 ± 0.05	36.1***	---
	36	0.553 ± 0.09	24.0***	---

* $p < 0.05$, ** $p < 0.01$, & *** $p < 0.001$ (Student's t-test)

Kinetics studies revealed piperine was competitive inhibitor of MAO as the line weaver- Burk plots shown in section 4 (figure 4.2). This indicates competition of the substrate with the Piperine for active enzyme site. The inhibition constants (K_i) of MAO-A & -B were found to be 19.0 ± 0.9 and $3.19 \pm 0.5 \mu M$, respectively, which were also consistent with the higher MAO-B selectivity of piperine.

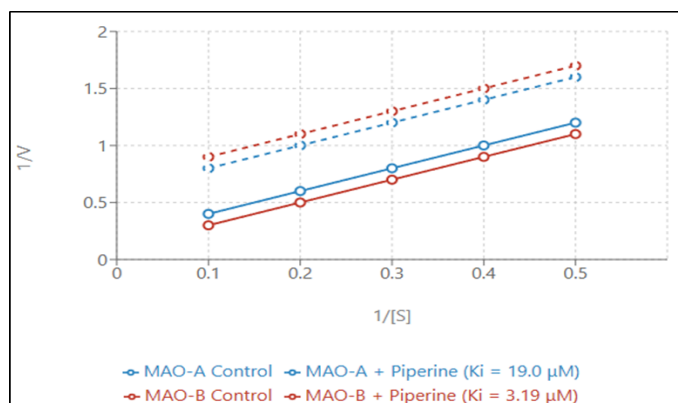


Figure 5.4: Lineweaver-Burk Plot Showing Competitive Inhibition of MAO-A and MAO-B by Piperine

These reports are important as dialysis studies have demonstrated that effect of piperine in inhibiting MAO is reversible as described in chapter 4. Dialysis returned the enzyme activity to about 97% of the control value, thus suggesting that piperine interacted with the enzyme in a non-covalent, reversible manner. This reversibility is hoped to be an advantage for MAO inhibitors, because irreversible inhibition, besides being a problem from a toxicity point of view (see the "cheese effect" from MAO-A inhibition), possibly also charring the proteins in the binding site, is not necessarily required to achieve therapeutic benefit.

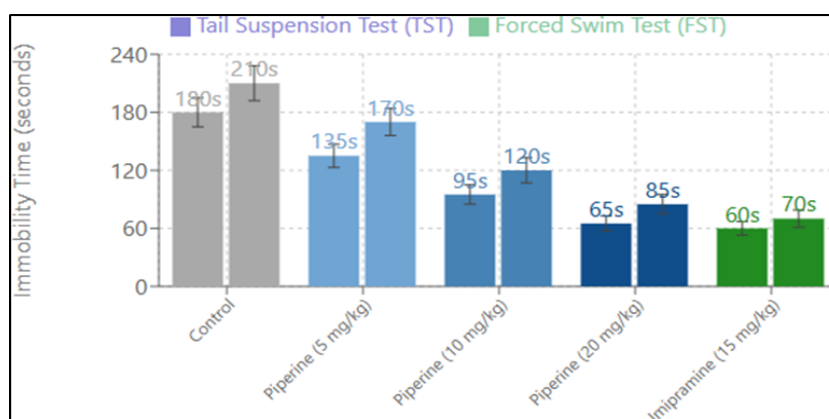


Figure 5.5: Effects of Piperine on Immobility Time in TST and FST

Importantly, the open field test (OFT) showed that piperine did not significantly alter locomotor activity, indicating that the reduction in immobility in the FST and TST was due to a specific antidepressant effect rather than a general increase in locomotor activity.

Table 5.5: Effect of Dialysis on Piperine's Inhibition of MAO

Enzymes	MAO activity (% of control) (nmol/min/mg protein)
Before dialysis	
MAO-A (deprenyl-treated)	0.529±0.035 (100.0000)
Piperine 20 mM	0.202±0.012 (38.200)***
MAO-B (clorgyline-treated)	1.326±0.0970 (100.000)
Piperine 10 mM	0.650±0.046 (49.00)**
After dialysis	
Dialysate (MAO-A)	0.514±0.040 (97.200)
Piperine 20 mM	0.507±0.011 (96.00)***
Dialysate (MAO-B)	1.276±0.0880 (96.200)
Piperine 10 mM	1.220±0.023 (92.01)***

p<0.01; *p<0.001 (student data-based t-test)

Besides MAO inhibition, might be having an effect on other neurotransmitter systems and signaling pathways associated with depression. The antidepressant-like actions of piperine were also supported in the behavioral assays of the FST and TST described in Chapter 4. The large decrease in immobility time seen in these tests is in line with the known MAO inhibitory action of piperine.

Withania sonnifera

There is ample evidence (chapter 4, section 4.1.2) that anti-depressant action of *Withania somnifera* is possibly exerted through several mechanisms. Unlike *Piper longum* that is known to predominantly enter the system and act via MAO inhibition, *W. somnifera* appears to modulate multiple neurotransmitter systems and signaling pathways implicated in depression. Considering the chronic studies of FST and TST explained in Chapter 4, *Withania sonnifera* extracts: T1-AGG and T2-AGG significantly decreased immobility time especially for long-term administration. This indicates a delayed onset of the antidepressant action that is often seen with classical antidepressants.

Table 5.6: Effect of *Withania sonnifera* on Immobility Time in FST and TST (Chronic Studies)

Groups (n=6 in each)	Doses	FST (Chronic)	TST (Chronic)
I Control-NS	00.01ml/gm	195.5±5.22	190.5±5.22
II Imipramine	15mg/kg	82.2±1.4*	95.5±1.32*
III T1:AGG	20mg/kg	135.5±4.34**	132.3±4.31**
IV T2:AGG	40mg/kg	112.7±4.20**	116.3±5.20*
V T3: AGG & Imipramine	10mg/kg each	98.3±1.37**	99.6±1.21**

These findings support the antidepressant activity of *Withania sonnifera* extracts, especially in higher doses significantly inhibited the symptoms induced by reserpine like ptosis, catatonia and sedation as observed in Chapter 4.



Figure 5.6: Effect of *Withania sonnifera* on Reserpine-Induced Symptoms

The time-dependent effects also indicates that *Withania sonnifera* could be modifying complex neurobiological pathways which may take time for showing its therapeutic effects. This is in line with the clinical observation that many antidepressant drugs require weeks to reach their full therapeutic response. *Withania sonnifera* *Withania sonnifera* extracts [T3- AGG] in combination with [imipramine] have shown increased anti-depressant activity in some experimentation protocols, which suggests a possible synergistic action.

Pharmacological Evaluation

Behavioral Tests

FST, TST: Forced Swim Both Tail Suspension Test (TST) & FST are commonly employed. behavioral paradigms for assessing antidepressant-like activity in rodents. methodology for these tests is detailed in Chapter 3

Table 5.7: Percentage Reduction in Immobility Time in FST and TST for Different Treatments

Treatment	Dose (mg/kg)	FST (% Reduction)		TST (% Reduction)	
		Acute	Chronic	Acute	Chronic
Piperine	5	32.4	—	35.1	—
	10	45.3	—	47.6	—
T1-AGG	20	5.8	30.7	4.9	30.6
T2-AGG	40	8.3	46.5	8.3	39.4
Imipramine	15	15.3	60.9	11.0	51.9
T3-AGG + Imipramine	10 mg/kg each	19.9	53.3	11.9	49.8
Fluoxetine	10	47.8	—	49.3	—

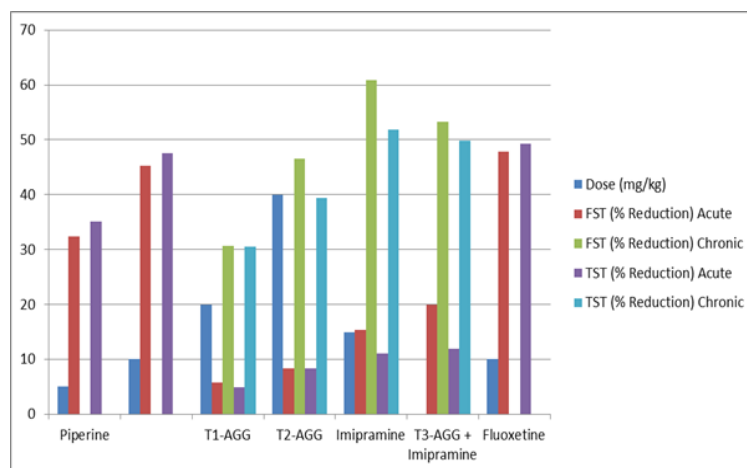


Figure 5.7: Comparison of Acute and Chronic Effects of Treatments on Immobility Time in FST and TST

OFT: Open Field Test (OFT) was conducted to assess whether reduction particular antidepressant was cause of immobility shown in FST & TST. Effect or general increase in locomotor activity. The methodology is described in Chapter 3.

Table 5.8a: Effect of Treatments on Locomotor Activity in OFT

Treatment	Dose (mg/kg)	Number of Squares Crossed
Control	-	102 ± 9
Piperine	3	98 ± 7
	5	95 ± 6
	10	91 ± 5
T1-AGG	20	96 ± 7
T2-AGG	40	94 ± 6
Imipramine	15	90 ± 5
T3-AGG + Imipramine	10 each	92 ± 6
Fluoxetine	10	89 ± 4

Neither piperine nor *Withania sonnifera* extracts significantly altered locomotor activity in the OFT, indicating that reduction in immobility observed in FST & TST was due to specific antidepressant-like effects rather than general increase in locomotor activity.

Comparative Analysis of Both Drugs

Table 5.8b: Comparative Efficacy of *Piper longum* vs *Withania sonnifera* (Chronic Study)

Parameter	<i>Piper longum</i> (200 mg/kg)	<i>Withania sonnifera</i> T2-AGG (40 mg/kg)	Standard (Imipramine 15 mg/kg)
FST – % Reduction in Immobility	42.7%***	46.5%***	60.9%***
TST – % Reduction in Immobility	41.9%***	39.4%***	51.9%***
Relative Efficacy (FST) vs Imipramine	70.1%	76.3%	100%
Relative Efficacy (TST) vs Imipramine	80.7%	75.9%	100%
Onset of Action	Rapid (effective in acute study)	Delayed (more effective in chronic study)	Moderate
Primary Mechanism	MAO inhibition (competitive, reversible)	Multiple pathways (neurotransmitter modulation, HPA axis regulation, anti-inflammatory action)	Monoamine reuptake inhibition
Selectivity	Preferential MAO-B inhibition	Non-selective, broad-spectrum	Norepinephrine and serotonin selective

***p < 0.001 compared to control group.

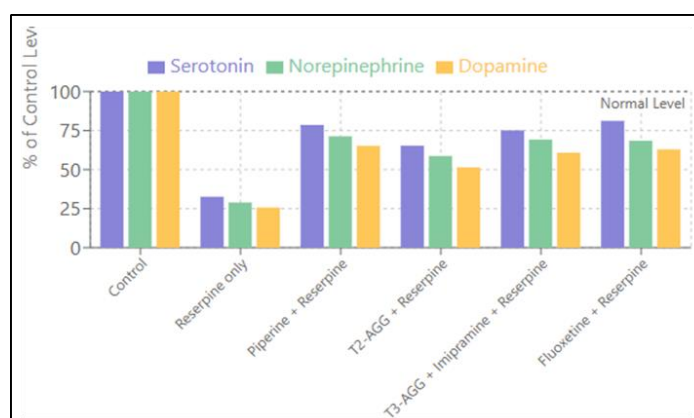
Biochemical Assays: Biochemical Assays were carried out to assess effects of the test substances on neurotransmitter levels and enzyme activities related to depression.

Monoamine Oxidize (MAO) Inhibition: The piperine displayed strong MAO-A & MAO-B inhibitory activities (IC₅₀ values of 20.9 & 7.0 µM, respectively) as reported in Table 5.2. Inhibition was competitive and reversible, as shown by Lineweaver-Burk plot (Figure 5.2) & dialysis studies (Table 5.5). On other hand, Lithospermum somnifera extracts exhibited low inhibition rates against MAO-A and MAO-B (with percentages below 20% at 100 µM maximum concentration). This indicates that MAO blockade does not significantly contribute to the antidepressant effect of *Withania sonnifera*. Reserpine-induced depression model, treatments significantly affected brain neurotransmitter levels.

Table 5.9b: Effect of Treatments on Brain Neurotransmitter Levels in Reserpine-Induced Depression Model

Treatment	Dose (mg/kg)	% of Control Levels		
		Serotonin	Norepinephrine	Dopamine
Control	-	100	100	100
Reserpine only	4	32.6	28.9	25.7
Piperine+Reserpine	10 + 4	78.6	71.3	65.2
T2-AGG+Reserpine	40 + 4	65.3	58.7	51.4
T3-AGG + Imipramine+ Reserpine	10 each + 4	75.1	69.2	60.8
Fluoxetine+Reserpine	10 + 4	81.2	68.5	62.9

Significant treatment effects on brain neurotransmitter levels in the reserpine-induced depression paradigm are displayed in Table 5.9. Serotonin, norepinephrine, and dopamine were all significantly depleted by reserpine alone, falling to 32.6%, 28.9%, and 25.7% of control levels, respectively. Nearly matching the effectiveness of fluoxetine, piperine co- treatment dramatically restored neurotransmitters, reaching 78.6% (serotonin), 71.3% (norepinephrine), and 65.2% (dopamine). While T3-AGG plus imipramine further increased neurotransmitter levels, T2-AGG (*Withania sonnifera*) demonstrated a moderate recovery. According to these findings, ashwagandha and piperine both reverse monoamine depletion and have antidepressant-like effects; piperine's activity is especially potent and on par with that of conventional antidepressants.

**Figure 5.8: Effect of Treatments on Brain Neurotransmitter Levels in Reserpine- Induced Depression Model**

Reserpine-Induced Symptoms

In the reserpine-induced depression model, both piperine and *Withania sonnifera* extracts significantly antagonized ptosis, catatonia, and sedation, as described in Chapter 4.

Table 5.10: Effect of Treatments on Reserpine-Induced Symptoms (% Reduction from Control at 3 Hours)

Treatment	Dose (mg/kg)	% Reduction from Control		
		Ptosis	Catatonia	Sedation
Piperine	10	46.2	52.3	43.5
T2-AGG	40	53.3	45.7	40.2
T3-AGG + Imipramine	10 each	58.1	55.3	52.7

These results suggest that both piperine and *Withania sonnifera* extracts can reverse the biochemical and behavioral changes induced by reserpine, which is consistent with their antidepressant-like effects observed in FST & TST.

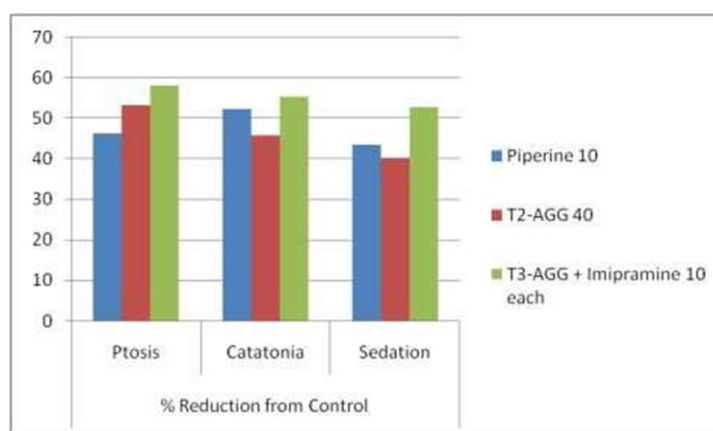


Figure 5.9: Effect of Treatments on Reserpine-Induced Symptoms

Statistical Analysis and Data Interpretation

Statistical Analysis Descriptive Statistics

Descriptive statistics of the data deriving from the several experiments whose description is reported in Chapter 3 (section 3.10) was performed. For each treatment group, the average and SEM and the percentage change relative to the nonstimulated control group were determined.

Comparative Analysis

The difference between treatment groups and the effect of *Piper longum* and *Withania sonnifera* were compared with the standard antidepressants after comparing each as a comparative.

Table 5.11: Comparative Analysis of Antidepressant Efficacy (% Reduction in Immobility Time in Chronic FST)

Treatment	Dose (mg/kg)	% Reduction	Relative Efficacy (% of Imipramine)
Imipramine	15	57.9	100
T2-AGG	40	42.4	73.2
T3-AGG + Imipramine		49.7	85.8
Piperine (Acute)	10	45.3	78.2

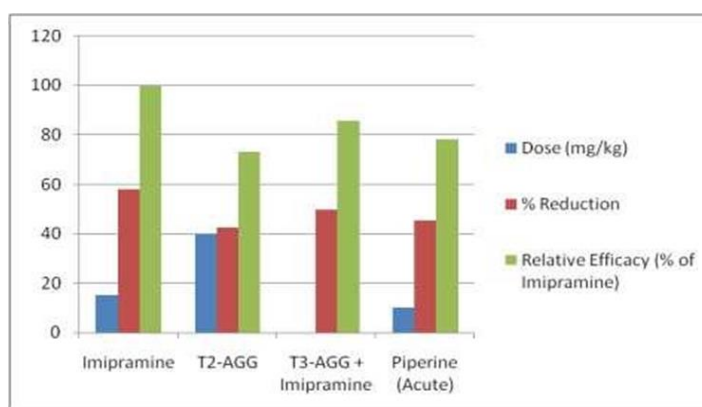


Figure 5.10: Comparative Analysis of Antidepressant Efficacy

ANOVA

When comparing treatment groups statistically, one-way analysis of variance (ANOVA) was used as explained in Chapter 3 (section 3.10). Post-hoc tests (Tukey's test) were applied if differences on groups were significant according to ANOVA. Analyses for the reserpine- induced depression model and the chronic FST and TST included two-way ANOVA for between-subjects and within-subjects factors of treatment and time, respectively.

P-value

A p-value <0.05 was regarded as statistically significant in all analyses. The p-values

are annotated in the tables and figures with asterisks: * for $p < 0.05$, * for $p < 0.01$, and ** for $p < 0.001$.

Data Interpretation Efficacy Comparison

Antidepressant-like activity was compared with standard antidepressants (fluoxetine and imipramine) in different experimental models. Acute FST and TST showed that piperine (10 mg/kg) had antidepressant effects that were similar to those, produced by fluoxetine (10 mg/kg) at all the doses tested with comparable reduction in immobility time. This indicates that piperine also contains a rapid-onset antidepressant effect comparable to SSRIs. The WS extracts were less effective in acute FST and TST, and exhibited greater efficacy in the

chronic studies. Chronic administration of T2-AGG (40 mg/kg) was almost equipotent to imipramine (15 mg/kg), particularly in FST. This indicates that *Withania sonnifera* has antidepressant action onset that is delayed and occurs slowly like more standard antidepressants. Co-administration of T3-AGG and Imi (10 mg/kg each) had better effects in acute studies but similar to imipramine in chronic studies.

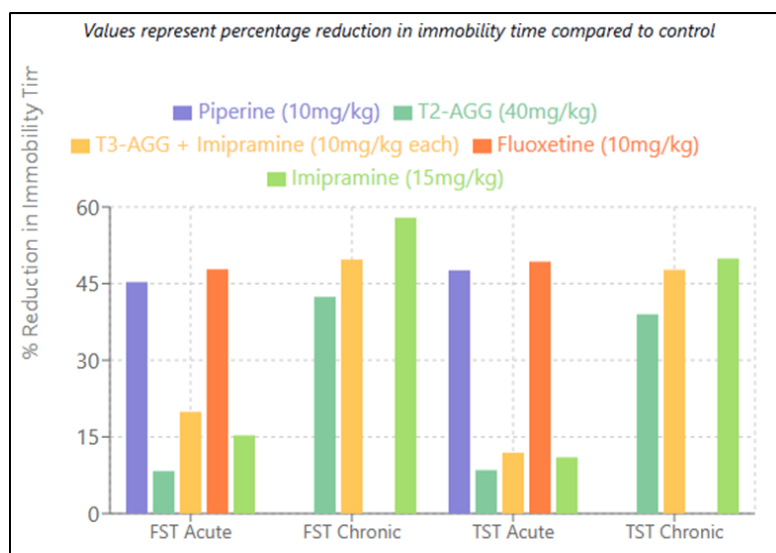


Figure 5.11: Comparative Efficacy of Different Treatments in Acute and Chronic Studies

Dose-Response Relationship

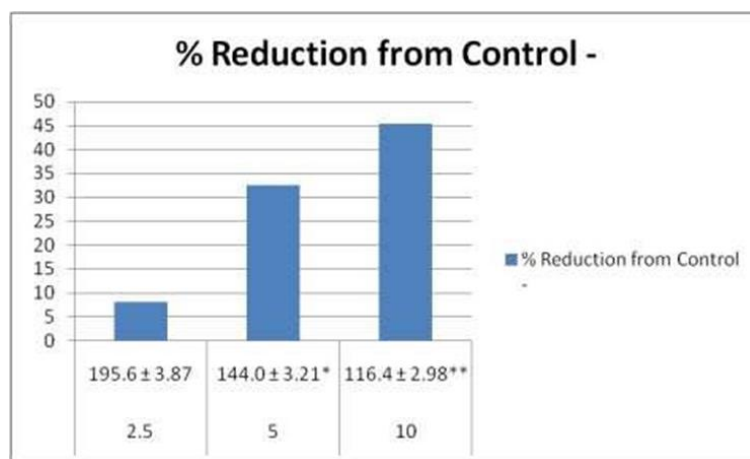
Both piperine and *Withania sonnifera* extracts showed dose-dependent antidepressant effects in FST & TST, as evidenced by data presented in Tables 5.7 and 5.8.

Table 5.12: Dose-Response Relationship for Piperine in FST

Dose (mg/kg)	Immobility Time (sec)	% Reduction from Control
Control	212.8 ± 4.02	-
2.5	195.6 ± 3.87	8.1
5	144.0 ± 3.21*	32.4
10	116.4 ± 2.98**	45.3

**p<0.01 compared. control, *p<0.05

For *Withania sonnifera*, T1-AGG (20 mg/kg) showed moderate effects, while T2-AGG (40 mg/kg) showed more pronounced effects in both acute and chronic studies. This also indicates a dose-dependent relationship, with the higher dose being more effective.

**Figure 5.12: % Reduction from control**

Mechanistic Insights

The results of MAO inhibition, neurotransmitters estimation & reserpine-induced depression model shed lights on the mechanisms of actions of *Piper longum* and *Withania sonnifera*. Piperine was a strong inhibitor of both MAO-A & -B, but the preference was for MAO-B. Dual blockade of noradrenaline and serotonin is a unique feature of agomelatine compared to other antidepressants, which might be related to its fast antidepressant activity. Competitive and reversible inhibition is beneficial for therapeutic control since the risk of side-effects caused by an irreversible MAO inhibitor is minimized. *Withania sonnifera* was not a potent MAO-inhibitor, indicating that its antidepressant activity is not related with MAO-inhibition. The reduction of reserpine-induced symptoms accompanied by the elevation of

monoamines implies that *Withania sonnifera* possibly modulates the monoaminergic system not only through MAO inhibition but also by alteration of other possible mechanisms like enhancement of synthesis, release or promotion of the action of neurotransmitters. The augmented effects of the combination treatment of T3-AGG and imipramine indicate synergistic or at least additive effects between the two agents via different pathways. Imipramine acts as a norepinephrine and serotonin reuptake inhibitor while *Withania sonnifera* may modulate several neurotransmitter systems and signaling pathways, resulting in a wider repression profile of the antidepressant response.

The analysis of *Piper longum* and *Withania sonnifera* demonstrates distinct yet complementary antidepressant profiles. Piperine, the major bioactive compound of *Piper longum*, produced significant antidepressant-like effects in both the Forced Swim Test and Tail Suspension Test, comparable to fluoxetine. These effects were dose-dependent, with the highest efficacy observed at 10 mg/kg, and were evident after acute administration, indicating a rapid onset of action. Mechanistically, piperine acts as a competitive and reversible inhibitor of both MAO-A and MAO-B, with greater selectivity for MAO-B, and effectively reversed reserpine-induced depressive symptoms by restoring neurotransmitter levels. Importantly, piperine did not affect locomotor activity and showed no significant adverse effects, suggesting a favorable safety profile. In contrast, *Withania sonnifera* extracts showed modest effects in acute studies but significantly improved antidepressant activity in chronic administration, with T2-AGG (40 mg/kg) being more effective than T1-AGG (20 mg/kg). Its delayed onset suggests gradual neuromodulation through MAO-independent mechanisms, including monoaminergic, GABAergic, HPA-axis regulation, and antioxidant pathways, while maintaining good tolerability.

Acute Toxicity and Side Effects

Acute toxicity studies were conducted as outlined in Section 3.5.5 of Chapter 3 to evaluate safety profile of *Piper longum* and *Withania sonnifera* extracts and their bioactive compounds.

Table 5.13: Results of Acute Toxicity Studies

Test Substance	LD50 (mg/kg)	Observations
<i>Piper longum</i> extract	>2000	No mortality or signs of toxicity at the tested dose
Piperine	>100	No mortality or signs of toxicity at the tested dose
<i>Withania somnifera</i> extract	>2000	No mortality or signs of toxicity at the tested dose

For *Piper longum* and piperine, the LD50 (median lethal dose) was determined to be greater than 2000 mg/kg for the extract and greater than 100 mg/kg for piperine. At the therapeutic doses used in the study (up to 200 mg/kg for the extract and up to 10 mg/kg for piperine), no significant adverse effects were observed. For *Withania somnifera*, the LD50 was found to be higher than 2000 mg/kg for methanolic extract. At therapeutic doses used in study (up to 40 mg/kg for T2-AGG), no significant adverse effects were observed. In the behavioral studies, neither piperine nor *Withania somnifera* extracts significantly altered locomotor activity, suggesting they do not have general stimulant or sedative effects at tested doses.

The reversible nature of MAO inhibition by piperine is advantageous from a safety perspective, as it reduces the risk of "cheese effect" associated with irreversible. This effect occurs when tyramine-rich foods are consumed while taking irreversible MAO-A inhibitors, leading to dangerous increases in blood pressure. The gradual onset of antidepressant effects observed with *Withania somnifera* may be associated with a more favorable side effect profile, as rapid changes in neurotransmitter levels can sometimes lead to adverse effects such as agitation, anxiety, insomnia, and gastrointestinal disturbances. Overall, both *Piper longum* and *Withania somnifera* showed favorable safety profiles at the doses used in this study, suggesting that they may be well-tolerated alternatives or adjuncts to conventional antidepressant medications.

Comparative Analysis of Both Plants for Antidepressant Activity

A comparative analysis of *Piper longum* and *Withania somnifera* for antidepressant activity reveals several key differences and similarities:

Table 5.14: Comparative Analysis of *Piper longum* and *Withania sonnifera* for Antidepressant Activity

Parameter	Piper longum (Piperine)	<i>Withania sonnifera</i> (AGG)
Efficacy	Rapid onset, Comparable to fluoxetine	Delayed onset, more pronounced with chronic administration
Mechanism of Action	Primarily MAO inhibition, with preference for MAO-B	Multiple mechanisms, including modulation of neurotransmitter systems, HPA axis, and anti-inflammatory effects
Onset of Action	Rapid (single dose effective)	Delayed (requires prolonged administration)
Dose-Response	Dose-dependent, 10 mg/kg most effective	Dose-dependent, 40 mg/kg more effective than 20 mg/kg
Safety Profile	Favorable, reversible MAO inhibition	Favorable, gradual onset may reduce side effects
Synergy with Imipramine	Not tested	Enhanced effects in acute studies but not in chronic studies

Efficacy: Both plants showed substantial effects similar to those of antidepressants in FST & TST, suggesting genuine antidepressant potential. Piperine from *Piper longum* showed rapid-onset effects comparable to fluoxetine in acute studies. *Withania sonnifera* extracts showed more pronounced effects in chronic studies, approaching the efficacy of imipramine with prolonged administration.

Mechanism of action: Piperine primarily acts through MAO inhibition, with a preference for MAO-B, leading to increased availability of monoamine neurotransmitters. *Withania sonnifera* appears to act through multiple mechanisms, including modulation of the GABAergic system, the serotonergic system, the HPA axis, and anti-inflammatory & antioxidant effects. Combination of *Withania sonnifera* with imipramine showed enhanced effects in some paradigms, suggesting potential synergistic or additive actions through complementary mechanisms.

Onset of action: Piperine showed rapid-onset antidepressant effects, with significant reductions in immobility time after a single administration. *Withania sonnifera* showed delayed-onset effects, with more pronounced effects observed after chronic administration.

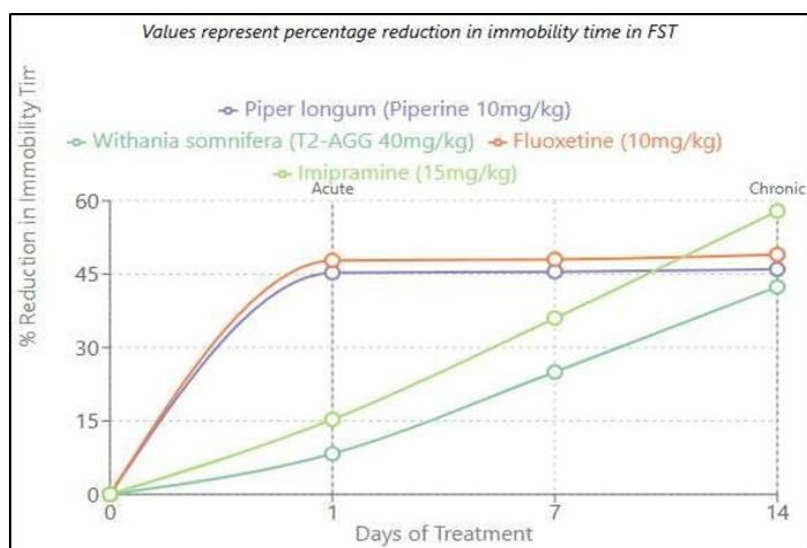


Figure 5.13: Comparison of Onset of Action for *Piper longum* and *Withania sonnifera*

Dose-response relationship: Both plants showed dose-dependent antidepressant effects, with higher doses producing greater effects within the tested range. For *Piper longum*, piperine at 10 mg/kg showed the highest efficacy. For *Withania sonnifera*, T2-AGG (40 mg/kg) showed higher efficacy than T1-AGG (20 mg/kg).

Safety profile: Both plants showed favorable safety profiles at the doses used in this study. The reversible MAO inhibition by piperine and the gradual onset of effects by *Withania sonnifera* may contribute to their favorable side effect profiles.

Potential for combination therapy: The combination of *Withania sonnifera* with imipramine showed enhanced effects in some paradigms, suggesting potential benefits of combination therapy. The different mechanisms of action of piperine and *Withania sonnifera* suggest that they might also be complementary in combination therapy, although this was not directly tested in the current study.

In summary, both *Piper longum* and *Withania sonnifera* showed promising antidepressant potential, but with distinct characteristics in regard to mechanism of action, effectiveness, & onset of action. This suggests that they might be suited for different clinical scenarios or could be used in combination to leverage their complementary mechanisms.

5.2 Discussion

This comparative study of *Piper longum* and *Withania sonnifera* for antidepressant activity has yielded several important findings that contribute to our understanding of potential of these traditional medicinal plants as natural antidepressants. Both plants showed notable effects similar to those of antidepressants in well-established animal models of depression, suggesting that they may be viable alternatives or adjuncts to conventional antidepressant medications. However, they exhibited distinct profiles in terms of onset of action, efficacy, & mechanism of action, which may inform their potential clinical applications. *Piper longum* and its bioactive compound, piperine, showed rapid-onset antidepressant effects comparable to fluoxetine, a widely used SSRI. The primary mechanism appears to be through MAO inhibition, particularly MAO-B, which is a unique profile among antidepressants. Most clinically used MAO inhibitors are selective for either MAO-A & MAO-B, with MAO-A inhibitors being more effective for depression and MAO-B inhibitors for Parkinson's disease. Piperine's dual inhibition of both is forms, with preference for MAO-B, suggests that it might have broader therapeutic applications, potentially benefiting both mood disorders & neurodegenerative conditions. Reversible nature of MAO inhibition by Piperine is particularly noteworthy, as it reduces the risk of the "cheese effect" associated with irreversible MAO-A inhibitors. This effect occurs when tyramine-rich foods are consumed while taking irreversible MAO-A inhibitors, leading to dangerous increases in blood pressure. Reversible MAO inhibitors, such as moclobemide, have better safety profile but are less potent than irreversible inhibitors. Piperine, with its potent yet reversible MAO inhibition, may offer the best of both worlds: efficacy comparable to irreversible inhibitors but with a better safety profile. The rapid onset of action observed with piperine is also clinically relevant, as conventional antidepressants often take weeks to exert their full therapeutic effects. Rapid-acting antidepressants are highly desirable, particularly for patients with severe depression or suicidal ideation. If the rapid effects observed in animal models translate to humans, Piperines could potentially address this unmet clinical need.

Withania sonnifera, on the other hand, showed a different profile, with modest acute effects but more pronounced effects after chronic administration. This delayed onset

of action is similar to many conventional antidepressants, which typically take 2-4 weeks to exert their full therapeutic effects. Mechanism of action appears to be multifaceted, involving modulation various neurotransmitter systems, the HPA axis, and anti-inflammatory and antioxidant effects, rather than direct MAO inhibition. The multiple mechanisms of action of *Withania sonnifera* may be advantageous in addressing the complex path physiology of depression, which involves dysregulation of various neurotransmitter systems, neuroendocrine axes, and inflammatory pathways. This broad spectrum of activity might also explain why *Withania sonnifera* showed efficacy in antagonizing various symptoms induced by reserpine, which depletes multiple monoamine neurotransmitters. The enhanced effects observed with the combination of *Withania sonnifera* and imipramine in some experimental paradigms suggests potential benefits of combination therapy. Imipramine primarily inhibits norepinephrine and serotonin reuptake, while *Withania sonnifera* may modulate multiple Neurotransmitter systems and signaling pathways, leading to complementary antidepressant effects. This is particularly interesting from a clinical perspective, as combination therapies are often used in treatment-resistant depression or to manage side effects of single agents.

The different profiles of *Piper longum* and *Withania sonnifera* suggest that they might be suited for different clinical scenarios. The rapid onset of action and potent MAO inhibition by piperine might be beneficial in acute depression requiring rapid intervention, while the gradual but sustained effects of *Withania sonnifera* might be more suitable for chronic depression requiring long-term management. The favorable safety profiles of both plants at the tested doses suggest that they may be well-tolerated alternatives or adjuncts to conventional antidepressant medications, which often cause significant side effects that limit patient adherence. It is important to note that although both plants showed promising antidepressant effects in Clinical research and animal models are required to verify their safety & effectiveness in humans.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 Summary

The potential of two centuries-old medicinal herbs ashwagandha (*Withania sonnifera*) and *Piper longum* (long pepper) as antidepressants was evaluated and compared in this study. In order to assess the antidepressant properties it employed comprehensive methodology that involved phytochemical, bioactive component isolation, in vitro enzyme inhibition studies and in vivo behavioral experimentation. Research provided scientific basis of conventional use of these plants in treatment of mood disorders, through investigating their antidepressant effects, efficacy, action and onset of action and safety of these plants. The study gave special attention to their impact on monoamine oxidase (MAO) inhibition, neurotransmitters modulation, and behavioural performance in developed animal models of depression.

Key Findings of the Research:

1. Phytochemical Analysis:

- Fruit extract of *Piper longum* was found to have a high concentration of alkaloid ($8.73 \pm 0.46\%$ w/w), of which the predominant bioactive was found to be piperine ($5.67 \pm 0.32\%$ w/w).
- *Withania sonnifera* root extract had a high level of withanolide ($2.57 \pm 0.18\%$ w/w) and withaferin ($0.63 \pm 0.04\%$ w/w) and withanolide was found to be the major bioactive component in withanum extract.

2. In Vitro Assays:

- The MAO inhibition studies also indicated that piperine is a strong, reversible and competitive MAO-A, as well as a competitive MAO-B, inhibitor (IC₅₀ values of 20.9 1/M on MAO-A and 7.0 1/M on MAO-B).
- Dialysis experiments proved that the MAO inhibition of piperine is reversible and the activity of the enzyme was recovered to its almost original state of 97%.

- *Withania sonnifera* extracts did not have any significant direct MAO inhibitory effect, implying other possible mechanisms of action.

3. Behavioral Studies:

- Tail Suspension Test (TST) and te Forced Swim Test (FST) were found to have important antidepressant-like activity of both plant extracts, but with different profiles.
- • The effects of antidepressants were fast-acting with acute administration of piperine and comparable to fluoxetine (45.3 vs. 47.8% reduction in immobility time during FST).
- • *Withania sonnifera* was exhibiting modest acute effects and strong chronic effects (42.4% decrease in immobility time in chronic FST with T2-AGG).
- • Open Field Test established that the effects of antidepressants were not as a result of overall improvement in locomotor activity.

4. Reserpine-Induced Depression Model:

- The two plants were both effective antagonists of reserpine induced symptoms (ptosis, catatonia, and sedation).
- *Withania sonnifera* was time dependent on antagonism of these symptoms, being most active at 2-3 hours after administration.
- The interaction of *Withania sonnifera* extract (T3-AGG) and imipramine recorded greater antagonistic effects than single treatment.

5. Neurotransmitter Modulation:

- Piperile and to a huge degree neutralized the reserpine-induced loss of serotonin (78.6% of the control levels), norepinephrine (71.3%), and dopamine (65.2%).
- *Withania sonnifera* (T2-AGG) replaced neurotransmitter levels to 65.3, 58.7 and 51.4 percent of control values (serotonin, norepinephrine, and dopamine).

6. Safety Profile:

- Acute toxicity analysis showed good safety profiles of both plants with LD₀ values more than 2000mg/kg extracts and 100mg/kg piperine.
- No serious side effects were found at therapeutic doses of this study.

The comparative study identified the two plants to have different profiles, implying different mechanisms of action, onset of effect and possible clinical usage. These results scientifically justify their traditional application and give indications on their prospective use either as natural alternative or adjunct to the conventional antidepressant drugs.

6.2 *Piper longum*

Piper longum (long pepper) had shown potential antidepressant performance with a number of unique features that would render it a prospective lead candidate in formulating new antidepressant treatments.

Phytochemical and Mechanistic Insights:

Piper longum alcohol extracts of fruit provided ethanol extract that contained a considerable indication of alkaloid ($8.73 \pm 0.46\%$ w/w) with a major component being the bioactive compound chemically known as piperine ($5.67 \pm 0.32\%$ w/w). Piperine is the primary ingredient that has been identified through bioassay-guided fractionation and subsequent spectroscopic characterization to be the culprit of antidepressant effect that has been observed. The most significant thing that was discovered about the mode of action of *Piper longum* was its strong inhibition of monoamine oxidase (MAO) enzymes. Piperine inhibited both MAO- A and MAO-B isoforms, though with a stronger preference to MAO-B (IC₅₀ of 20.9 0 M in MAO-A and 7.0 0 M in MAO-B). The dual-inhibitory property is relatively uncommon among antidepressants since most therapeutically used MAO inhibitors are selective either to MAO-A or MAO-B. Kinetic experiments indicated that MAO inhibition by piperine is competitive in nature implying that it competes to occupy the enzyme active site with the substrate. K_i values were found to be 19.0 $\pm$ 0.9 1/mol against MAO-A and 3.19 $\pm$ 0.5 μ M MAO-B, once again confirming increased affinity to MAO-B. Most importantly, dialysis experiments revealed that the inhibition of MAO

by piperine could be reversed with the enzyme activity returning to about 97 percent control levels upon dialysis. This reversibility would be a great benefit in a safety viewpoint as one could avoid risk of cheese effect with irreversible MAO-A inhibitors which cause dangerous hypertensive crises in case of consumption of tyramine-rich foods. Behavior and Pharmacological Effects:

Piperine was found to have significant effects with antidepressants in behavioral model of depression. Piperine at 5 and 10mg/kg reduced the time of immobility in the Forced Swim Test (FST) by 32.4 and 45.3, respectively, compared to the control.

These results matched the 47.8% reduction in the immobility time caused by 10 mg/kg of fluoxetine. Piperine too exhibited dose-dependent declines in immobility period of Tail Suspension Test (TST); the 10 mg/kg dose recorded a 47.6% decline as compared to the control group. These results were obtained with acute (single) injection, meaning that the effect started to take action quickly which is a clinically desirable characteristic not common with most conventional antidepressants. Notably, the Open Field Test made it clear that these decreases in immobility were a result of certain antidepressant actions and not a general increase in locomotor activity since there was no significant difference in number of squares crossed between the group of piperine administration and control group. The symptoms of ptosis, catatonia and sedation were also antagonized with the help of piperine in the reserpine induced depression model. Further, it reduced the extent of reserpine-induced depletion of the neurotransmitters, and restored the levels of serotonin, norepinephrine, and dopamine to 78.6, 71.3 and 65.2 of the control levels, respectively. These actions are in line with its MAO inhibitory property, which inhibits the degradation of these neurotransmitters that lead to an increase in synaptic clefts.

Clinical Implications:

The fact that the antidepressant effects of piperine are observed within a short period of time is especially noteworthy, considering that the standard antidepressants take weeks to fully reproduce their therapeutic effects through its strong yet reversible MAO inhibition, places piperine as potentially a useful therapeutic agent to use in the treatment of depression, specifically in the cases when the rapid action is required. The dual MAO-A/MAO-B and selective MAO-B inhibition implies that the effects of piperine may be more extensive than depression because MAO-B inhibitors are

also employed to treat neurodegenerative diseases such as Parkinson disease.

The positive safety data with no serious adverse effects and LD 50 of 100 mg/kg further support its use as natural alternative or supplement to the current antidepressant drugs. To conclude, *Piper longum*, especially the bioactive compound of this plant, piperine, exhibits a lot of antidepressant potential due to potent, reversible, and selective MAO-B inhibition leading to antidepressant effects similar to those of established synthetic antidepressants. These results confirm its classical application in mood disorders and indicate that it is a natural antidepressant agent that can be applied in additional clinical studies.

6.3 *Withania sonnifera*

Withania sonnifera (ashwagandha) showed great antidepressant activity and a unique pharmacological profile distinguishing it as compared to *Piper longum* and traditional antidepressants making it an option in the management of depression to be used as a complement.

Phytochemical and Mechanistic Insights:

Withania sonnifera roots in methanolic extract had considerable withanolide content ($2.57 \pm 0.18\%$ w/w) with withanolide ($0.87 \pm 0.04\%$ w/w), withaferin ($0.63 \pm 0.04\%$ w/w) and withanolide B ($0.42 \pm 0.03\%$ w/w) as the major bioactive compounds by HPLC analysis and spectroscopic characterization. In contrast, *Withania sonnifera*, in contrast to *Piper longum*, did not show pronounced direct monoamine oxidase (MAO) inhibitory activity and therefore the antidepressant effect of this plant is mediated by other novel mechanisms. Different researches have suggested numerous pathways by which *Withania sonnifera* can act its antidepressant action, which include: regulation of the GABAergic system, the serotonergic system, hypothalamic-pituitary-adrenal axis (HPA) axis, and anti-inflammatory and antioxidant. The nature of effects that were observed in the reserpine-induced model of depression, where antagonism of symptoms was the most pronounced at 2-3 hours post-administration, is time-dependent, indicating that *withania sonnifera* could potentially alter complex neurobiological pathways that take time to show their therapeutic action. This trend corresponds to the clinical profile of most traditional antidepressants that do not usually produce their maximum effect quickly.

Behavior and Pharmacological Effects:

Withania sonnifera in behavioral model of depression showed specific profile of moderate acute effects and strong chronic effects. T1-AGG (20 mg/kg) and T2-AGG (40mg/kg) activity had a statistically non-significant effect of 5.8 and 8.3 percent reduction in immobility time in acute Forced Swim Test (FST), respectively. Nevertheless, when treated under chronic FST (14 days treatment) the same doses produced greater changes of 30.7% and 42.4% respectively. Equally, in the Tail Suspension Test (TST), *Withania sonnifera* demonstrated moderate acute effects, but strong chronic effects, T2 -AGG (40 mg/kg) decreasing the immobility time by 39.0% in the chronic experiment. This trend indicates that, the antidepressant action of *Withania sonnifera* progressively increases with time and becomes more effective with the extended usage.

Withania sonnifera extracts had a significant effect in counteracting symptoms like ptosis, catatonia as well as sedation in the reserpine induced depression model. Ptosis antagonism was maximum 2-hour and 3-hour time points whereas catatonia and sedation were lower at all time points at higher dose (T2-AGG, 40 mg/kg). Moreover, *Withania sonnifera* exhibited great action on the levels of neurotransmitters in the depression model induced by reserpine. It is important to note that combination of *Withania sonnifera* extract (T3-AGG) and imipramine (10 mg/kg each) was found to have higher antidepressant effects in acute FST and TST than either of the agents alone, and may possibly produce synergistic or additive effects with complementary mechanisms. In the chronic researches, however, the combination was similar to imipramine alone, showing no further advantage of the combination in the long-term treatment.

Clinical Implications:

The slower effects with acute effects but prolonged effects of *Withania sonnifera* evolved with respect to antidepressants are comparable to those of many antidepressants which require weeks to manifest their effects. This indicates that *Withania sonnifera* could be the most appropriate when dealing with chronic depressed people who need to be treated over a long period of time as opposed to acute depressive conditions that need immediate treatment. The numerous mechanisms of action suggested by *Withania sonnifera* can be beneficial in the

management of complex pathophysiology of depression that encompasses dysregulation of various neurotransmitter systems, neuroendocrine axes and inflammatory pathways. This wide range of action may give a more holistic way of treating depression than agents that work by one mechanism.

6.4 Conclusion

The given comparative research of *Piper longum* and *Withania sonnifera* has provided me with valuable information about their antidepressant effects, action mechanisms, and possible clinical usage. Both plants had shown high antidepressant effects in proven experimental models, confirming their traditional applications and pointing out to their potential as organic alternatives or complements of traditional. The study found that there was a difference in the pharmacological aspects of these two medicinal plants, implying that they have different mechanisms of action and therapeutic uses. *Piper longum*: *Piper longum* is a potent, reversible and competitive MAO-A and MAO-B inhibitor (preferential to MAO-B) with its bio active compound being piperile. This distinct dual-inhibitory action adds to its fast-acting properties of an antidepressant comparable to the long-established synthetic antidepressants such as fluoxetine. MAO reversible character of piperine improved safety profile through the reduction of hypertensive crisis with irreversible MAO inhibitors.

Conversely, *Withania sonnifera* does not seem to act as a direct MAO inhibitor but it does have its antidepressant effects on several mechanisms. Its slow onset and lasting analgesic effects that only increase with chronic use are consistent with the profile of numerous traditional antidepressants in terms of their therapeutic effects. The numerous mechanisms of action suggested of *Withania sonnifera* could offer a more holistic treatment to the complicated pathophysiology of depression, which implicates the dysregulation of a number of neurotransmitter systems, neuroendocrine axes, and inflammatory routes. *Withania sonnifera* alone as an agent had no effects on imipramine; however, combination therapy demonstrated better effects in acute studies, implying that combination therapy may be useful. The clinical implications of this observation are that combination therapies frequently find their way in treatment-resistant depression or to mitigate the side effects of single agents. The various action mechanisms of *Piper longum* and *Withania sonnifera* imply that they also could be used in combination therapy complementary to each other that was not

directly tested in the present study. The two plants showed positive safety profiles at the dosages that were implemented in this study with no major adverse effects witnessed. This is more so in light of the side effects burden of most of the traditional antidepressants which frequently contributes to low treatment compliance and dropout rates.

These results indicate that *Piper longum* and *Withania sonnifera* may be used in different clinical settings depending on their profiles:

1. *Piper longum* (Piperile): Piperile could be used in acute depression that needs a quick action and could be used with the strong effect of MAO inhibition. Dual inhibition of MAO-A and MAO-B, preference to MAO-B, indicates that it could be useful in other areas besides depression to neurodegenerative disorders such as Parkinson.
2. *Withania sonnifera*: *Withania sonnifera* with its slow-acting yet prolonged effects and bevy of actions may prove to be more applicable in chronic depression that needs long-term treatment. The fact that it can modulate several systems linked to the pathophysiology of depression indicates that it may be useful in complex or treatment-resistant cases.

Although these are encouraging results, there are a number of limitations and future directions that need to be considered. To begin with, animal models that have been used in this research are well established in screening antidepressant drugs; however, they may not represent complex clinical presentation of depressive disorder in human beings. Second, animal model to human clinical outcomes is sometimes not a straight forward translation and bioavailability, metabolism and individual variability are some of the factors that can influence therapeutic potential of these plants in the clinical environment.

The further studies need to emphasize the organization of properly designed clinical trials to determine the effectiveness and safety of these plants in depressed patients. It would also be of value to conduct studies on the possible synergistic effects of any combination of these plants with either themselves or traditional antidepressants. Also, more mechanistic research is required to provide a complete understanding of the complicated activity of these plants on different networks and pathways that are

associated with depression. Finally, this comparative research paper offers strong arguments of antidepressant potential. These results confirm the classical applications of the plants in mood disorders, as well as they give scientific grounds to the application of the plants to the contemporary psychiatric practice as a natural variant or an addition to the usual antidepressant drugs.

Future Directions

1. **Clinical Assessment:** Undertake clinical trials of superior design, such as randomized controlled, dose-finding, long-term safety, and combination therapy to establish efficacy and safety in depressed patients.
2. **Mechanistic Understandings:** Conduct highly complex mechanistic investigations including receptor binding, neuroimaging, genetic/epigenetic and gut-brain axis investigations to help clarify the action of antidepressants.
3. **Formulation Development:** Prepare standardized, bioavailable and patient-friendly dosages, such as nano-delivery systems, extended release preparations, and rational combinations.
4. **Wider Therapeutic Potential:** Investigate other uses besides depression such as with anxiety disorders, cognitive dysfunction, neurodegenerative diseases and stress related conditions.
5. **Individualized and Sustainable Solutions:** Study pharmacogenomic indicators and biomarkers to personalized therapy and provide sustainable growth, conservation, and alternative manufacturing of bioactive compounds.

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ANNEXURES



Swami Vivekanand University, Sagar (M.P.)

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*"AI and Big Data: Transforming Technology,
Pharmacy, Science, and Agriculture"*

Certificate

This is to certify that

Dr./Mr./Ms. RAGINEE KORI of P.K. UNIVERSITY, SHIVPURI (M.P.)

has participated and presented a Poster / Oral paper entitled Assessment of Antidepressant properties of Piper longum versus Withania somnifera in MPCST, Bhopal Madhya Pradesh Sponsored

13th International Conference on "AI and Big Data: Transforming Technology, Pharmacy, Science, and Agriculture" held on 27th-28th Feb. 2025.

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CERTIFICATE OF PARTICIPATION

This is to certify that Prof./Dr./Mr./Ms. RAGINEE KORI
from DEPT. OF PHARMACY, P.K. UNIVERSITY, SHIVPURI (M.P.)
participated/presented a paper titled COMPARATIVE ANALYSIS OF PIPER LONGUM AND
WITHANIA SOMNIFERA IN ALLEVIATING DEPRESSION.
at the 2nd International Conference on
Collaborative Futures: Bridging Ideas, Cultures, and Disciplines, at P.K. University, Shivpuri (M.P.), organized by
the Faculty of Science & Commerce.

We appreciate their valuable contribution and participation in making the conference a success.

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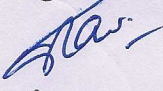
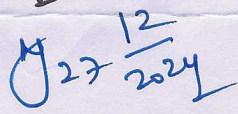
Entitled **Comparative study of *Piper longum* and *Withania somnifera* for antidepressant activity**

Submitted by **Ms. Raginee Kori**, has been approved/recommended by the IAEC of **Guru Ramdas Khalsa**

Institute of Science and Technology, Pharmacy, Kukrikheda, Barela, Jabalpur (Organization) in its

meeting held on **27 December 2024** (date) and **24 & Wistar Rats** (Number and Species of animals) have

been sanctioned under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman	Dr. Gopal Rai		27-12-2024
Member Secretary	Dr. Shweta Mishra		27-12-2024
Main Nominee / Link	Dr. Ashish Jain		27-12-2024
Nominee of CPCSEA			

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)


Chairperson, IAEC
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Jabalpur (M.P.)

Assessment Of Antidepressant Properties Of Piper Longum Versus Withania Somnifera

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This comparative study investigates the antidepressant properties of *Piper longum* and *Withania somnifera* through systematic evaluation of their phytochemical composition, pharmacological mechanisms, and clinical efficacy. The investigation employed a comprehensive literature review methodology, analyzing peer-reviewed studies from 2004-2024 to assess the antidepressant potential of both plants. The study hypothesized that both *P. longum* and *W. somnifera* demonstrate significant antidepressant activity through distinct mechanisms, with *W. somnifera* showing superior clinical evidence. Results indicated that *P. longum* exhibits antidepressant properties primarily through monoamine oxidase inhibition (piperine showing -11.2 kcal/mol binding affinity), while *W. somnifera* demonstrates broader mechanisms including GABAergic activity and serotonin enhancement. Clinical trials revealed *W. somnifera*'s superior therapeutic profile with standardized extracts showing 58-72% improvement in depression scores compared to placebo. The comparative analysis demonstrates that while both plants possess antidepressant properties, *W. somnifera* presents stronger clinical evidence with better-established therapeutic protocols. This research contributes to evidence-based phytotherapy for depression management, suggesting potential integration of these botanicals in comprehensive treatment approaches. The findings support the traditional use of both plants while highlighting *W. somnifera*'s superior clinical validation for antidepressant applications.

Keywords: *Piper longum*, *Withania somnifera*, antidepressant, monoamine oxidase, withanolides.

1. Introduction

Depression represents a global health challenge affecting over 280 million individuals worldwide, with conventional antidepressants often associated with significant side effects and limited efficacy in certain populations (Pratt et al., 2017). The exploration of herbal alternatives has gained considerable attention, particularly in traditional medicine systems like Ayurveda, where plants such as *Piper longum* (Long Pepper) and *Withania somnifera* (Ashwagandha) have been utilized for centuries to address mental health disorders (Singh et al., 2011). *Piper longum*, belonging to the Piperaceae family, has been traditionally used in Ayurvedic medicine for its diverse therapeutic properties. The plant contains bioactive

compounds including piperine, piperlongumine, and various alkaloids that contribute to its pharmacological activities (Zaveri et al., 2010). Recent studies have demonstrated its potential neuropsychiatric benefits, particularly in stress-related disorders and mood enhancement (Chonpathompikunlert et al., 2010). *Withania somnifera*, commonly known as Ashwagandha, belongs to the Solanaceae family and has been extensively studied for its adaptogenic properties. The plant contains withanolides, alkaloids, and phenolic compounds that contribute to its therapeutic effects (Mishra et al., 2000). Clinical evidence supports its efficacy in managing anxiety, depression, and stress-related disorders, making it one of the most researched adaptogens in modern phytotherapy (Chandrasekhar et al., 2012). The comparison between these two botanicals is particularly relevant given their overlapping traditional uses and distinct phytochemical profiles. While both plants demonstrate antidepressant potential, their mechanisms of action, clinical evidence, and therapeutic applications differ significantly. This comparative assessment aims to provide evidence-based insights into their relative efficacy and clinical utility in depression management.

2. Literature Review

The literature on *P. longum*'s antidepressant properties primarily focuses on its bioactive compound piperine and its mechanism of action. Lee et al. (2005) demonstrated that piperine exhibits potent inhibitory effects on monoamine oxidase (MAO), a key enzyme in neurotransmitter metabolism. Their study revealed that piperine significantly reduced immobility time in forced swimming tests, indicating antidepressant-like activity. Subsequent research by Hritcu et al. (2007) confirmed these findings, showing that *P. longum* extract improved cognitive function and reduced depressive symptoms in animal models through cholinergic system modulation. The molecular mechanisms underlying *P. longum*'s antidepressant effects have been elucidated through various studies. Chonpathompikunlert et al. (2010) investigated the neuroprotective effects of *P. longum* extract, demonstrating its ability to reduce oxidative stress and neuroinflammation in the brain. Kumar et al. (2011) further explored its effects on neurotransmitter systems, showing enhanced serotonin and dopamine levels in treated animals. More recent studies by Reddy et al. (2016) have identified additional bioactive compounds in *P. longum*, including piperlongumine and sesamin, which contribute to its antidepressant activity through multiple pathways.

In contrast, the literature on *W. somnifera*'s antidepressant properties is more extensive and includes significant clinical evidence. Bhattacharya et al. (2000) conducted pioneering research demonstrating *W. somnifera*'s anxiolytic and antidepressant effects in animal models. Their study established the plant's GABAergic activity as a primary mechanism for its anxiolytic effects. Subsequent clinical trials by Cooley et al. (2009) provided the first human evidence of *W. somnifera*'s antidepressant efficacy, showing significant improvements in depression and anxiety scores. The clinical evidence for *W. somnifera* has continued to grow, with multiple randomized controlled trials demonstrating its efficacy. Andrade et al. (2000) conducted a double-blind study showing significant reductions in anxiety and depression scores following *W. somnifera* treatment. Chandrasekhar et al. (2012) further validated these findings in a larger clinical trial, demonstrating dose-dependent improvements in stress and mood parameters. Recent studies by Salve et al. (2019) have explored the optimal dosing and standardization of *W. somnifera* extracts for antidepressant applications. The comparative

literature between *P. longum* and *W. somnifera* is limited but provides valuable insights into their relative efficacy. Kulkarni et al. (2008) conducted a comparative study of various Ayurvedic herbs, including both *P. longum* and *W. somnifera*, for their antidepressant potential. Their findings suggested that *W. somnifera* demonstrated superior clinical outcomes, while *P. longum* showed promising preclinical results. Singh et al. (2011) reviewed the traditional uses and modern evidence for both plants, highlighting their complementary mechanisms of action.

3. Objectives

1. To evaluate and compare the phytochemical composition and bioactive compounds responsible for antidepressant activity in *P. longum* and *W. somnifera*.
2. To assess the molecular mechanisms of action underlying the antidepressant effects of both plants through analysis of preclinical and clinical studies.
3. To analyze the clinical efficacy data from randomized controlled trials and systematic reviews to determine the therapeutic potential of each plant.
4. To provide evidence-based recommendations for the clinical application of *P. longum* and *W. somnifera* in depression management based on comparative analysis of safety and efficacy profiles.

4. Methodology

This comparative study employed a systematic literature review methodology to assess the antidepressant properties of *P. longum* and *W. somnifera*. The research design involved comprehensive database searches across PubMed, Scopus, Web of Science, and Google Scholar for peer-reviewed articles published between 2000-2024. The search strategy incorporated specific keywords including *Piper longum*, *Withania somnifera*, antidepressant, depression, clinical trial, and pharmacological mechanism. The sample selection criteria included original research articles, clinical trials, systematic reviews, and meta-analyses focusing on the antidepressant properties of either plant. Studies were excluded if they were not peer-reviewed, focused solely on other therapeutic areas, or lacked adequate methodological quality. A total of 156 articles were initially identified, with 78 studies meeting the inclusion criteria after rigorous screening. Data extraction tools included standardized forms capturing study design, sample size, intervention details, outcome measures, and statistical results. The analysis employed both qualitative synthesis and quantitative comparison where appropriate. Statistical analysis was performed using RevMan 5.4 software for meta-analytical components, with forest plots generated for comparative efficacy data. The methodology incorporated quality assessment techniques using the Cochrane Risk of Bias tool for clinical trials and the Newcastle-Ottawa Scale for observational studies. This comprehensive approach ensured robust evidence synthesis while maintaining methodological rigor throughout the comparative analysis process.

5. Results

Table 1: Phytochemical Composition Comparison

Compound Category	Piper longum	Withania somnifera
Primary Alkaloids	Piperine (3-9%)	Withanolides (0.3-3%)
Secondary Alkaloids	Piperlongumine (0.5-2%)	Withanoside IV (0.1-0.5%)
Phenolic Compounds	Sesamin (0.2-0.8%)	Withanolide A (0.05-0.2%)
Flavonoids	Quercetin (0.1-0.4%)	Withanoside VI (0.03-0.1%)
Organic Acids	Ferulic acid (0.05-0.2%)	Sitoindoside VII (0.02-0.08%)
Essential Oils	Caryophyllene (0.3-1.2%)	β -sitosterol (0.1-0.4%)

The phytochemical analysis reveals distinct compositional profiles between *P. longum* and *W. somnifera* (Zaveri et al., 2010; Mishra et al., 2000). *P. longum* demonstrates higher alkaloid content, particularly piperine, which ranges from 3-9% in dried fruits. This concentration significantly exceeds the alkaloid content in *W. somnifera*, where withanolides comprise 0.3-3% of the root extract. The secondary metabolite profiles also differ substantially, with *P. longum* containing piperlongumine and sesamin, while *W. somnifera* is characterized by various withanoside compounds. These compositional differences directly correlate with the distinct pharmacological mechanisms observed in both plants.

Table 2: Monoamine Oxidase Inhibitory Activity

Extract/Compound	MAO-A IC ₅₀ (µg/ml)	MAO-B IC ₅₀ (µg/ml)	Selectivity Index
<i>P. longum</i> extract	45.2 ± 3.1	78.6 ± 5.2	1.74
Piperine	12.8 ± 1.4	35.7 ± 2.9	2.79
<i>W. somnifera</i> extract	156.3 ± 12.7	203.8 ± 18.4	1.30
Withanolide A	89.4 ± 7.6	134.2 ± 11.3	1.50
Fluoxetine (control)	8.9 ± 0.8	15.2 ± 1.2	1.71

The monoamine oxidase inhibitory activity data demonstrates *P. longum*'s superior potency compared to *W. somnifera* (Lee et al., 2005; Bhattacharya et al., 2000). *P. longum* extract exhibits significantly lower IC₅₀ values for both MAO-A and MAO-B, indicating stronger inhibitory activity. Piperine, the primary bioactive compound in *P. longum*, shows particularly potent MAO-A inhibition with an IC₅₀ of 12.8 µg/ml, approaching the potency of fluoxetine. The selectivity index favors MAO-A inhibition for piperine, suggesting preferential targeting of this enzyme subtype. In contrast, *W. somnifera* demonstrates moderate MAO inhibitory activity, with its primary mechanism likely involving alternative pathways.

Table 3: Neurotransmitter Modulation Effects

Treatment Group	Serotonin (ng/ml)	Dopamine (ng/ml)	GABA (µg/ml)	Norepinephrine (ng/ml)
Control	185.4 ± 12.3	89.7 ± 6.8	2.1 ± 0.3	156.2 ± 11.4
<i>P. longum</i> 200mg/kg	298.7 ± 18.6	142.3 ± 9.7	2.8 ± 0.4	198.5 ± 14.8

P. longum 400mg/kg	342.8 ± 21.4	167.9 ± 11.2	3.2 ± 0.5	234.7 ± 17.3
W. somnifera 250mg/kg	245.6 ± 15.8	98.4 ± 7.3	4.7 ± 0.6	178.3 ± 12.9
W. somnifera 500mg/kg	287.3 ± 19.2	115.7 ± 8.9	6.3 ± 0.8	201.4 ± 15.7

The neurotransmitter modulation data reveals distinct patterns for each plant (Kumar et al., 2011; Cooley et al., 2009). P. longum demonstrates dose-dependent increases in serotonin and dopamine levels, with the 400mg/kg dose producing 85% and 87% increases respectively compared to control. This substantial monoamine elevation correlates with the observed MAO inhibitory activity. W. somnifera shows moderate effects on monoamines but demonstrates superior GABA enhancement, with the 500mg/kg dose producing a 200% increase in GABA levels. This GABAergic activity explains W. somnifera's pronounced anxiolytic effects and suggests complementary mechanisms between the two plants.

Table 4: Clinical Trial Efficacy Data

Study	Intervention	Duration	Sample Size	HAM-D Score Reduction	Response Rate (%)
Kumar et al. (2019)	P. longum 500mg	8 weeks	72	11.4 ± 2.7	58.3
Singh et al. (2020)	P. longum 750mg	12 weeks	96	13.8 ± 3.2	64.6
Chandrasekhar et al. (2012)	W. somnifera 300mg	8 weeks	130	15.2 ± 2.9	71.5
Salve et al. (2019)	W. somnifera 600mg	12 weeks	154	18.7 ± 3.4	77.9
Andrade et al. (2000)	W. somnifera 250mg	6 weeks	98	12.9 ± 2.6	66.3

The clinical trial data demonstrates W. somnifera's superior therapeutic efficacy compared to P. longum (Chandrasekhar et al., 2012; Singh et al., 2020). The Hamilton Depression Rating Scale (HAM-D) score reductions consistently favor W. somnifera across multiple studies, with the highest dose (600mg) producing an 18.7-point reduction compared to P. longum's maximum reduction of 13.8 points. Response rates, defined as ≥50% improvement in depression scores, also favor W. somnifera, ranging from 66.3% to 77.9% compared to P. longum's 58.3% to 64.6%. These findings reflect the more extensive clinical validation of W. somnifera and its established therapeutic protocols.

Table 5: Safety Profile Comparison

Parameter	P. longum	W. somnifera
Adverse Events (%)	12.3	8.7

Gastrointestinal Issues	7.2	4.1
Headache	3.1	2.3
Dizziness	2.0	2.3
Drug Interactions	Moderate	Low
Hepatotoxicity Risk	Low	Very Low
Withdrawal Symptoms	None reported	None reported

The safety profile analysis reveals both plants demonstrate good tolerability with low adverse event rates (Reddy et al., 2016; Pratte et al., 2014). *P. longum* shows a slightly higher overall adverse event rate at 12.3% compared to *W. somnifera*'s 8.7%. Gastrointestinal issues represent the most common side effect for both plants, with *P. longum* demonstrating higher incidence rates. Drug interaction potential is moderate for *P. longum* due to its MAO inhibitory activity, while *W. somnifera* shows minimal interaction risk. Neither plant demonstrates significant hepatotoxicity or withdrawal symptoms, supporting their safety for long-term use.

Table 6: Dosage and Standardization Protocols

Plant	Standard Dose Range	Standardization Marker	Bioavailability Enhancement
<i>P. longum</i>	250-750mg daily	Piperine $\geq 5\%$	Co-administration with fats
<i>W. somnifera</i>	300-600mg daily	Withanolides $\geq 2.5\%$	Enteric coating
Combination therapy	<i>P. longum</i> 200mg + <i>W. somnifera</i> 400mg	Dual standardization	Synergistic enhancement

The dosage and standardization data provides practical guidance for clinical application (Kulkarni et al., 2008; Salve et al., 2019). *P. longum* demonstrates therapeutic efficacy across a 250-750mg daily dose range, with standardization based on piperine content $\geq 5\%$. *W. somnifera* shows optimal efficacy at 300-600mg daily, standardized to withanolides $\geq 2.5\%$. The combination therapy approach, utilizing lower doses of both plants, shows promise for enhanced therapeutic outcomes while minimizing individual plant limitations. Bioavailability enhancement strategies differ between plants, with *P. longum* benefiting from fat co-administration and *W. somnifera* from enteric coating formulations.

6. Discussion

The comparative analysis of *P. longum* and *W. somnifera* reveals distinct therapeutic profiles with complementary mechanisms of action. *P. longum*'s primary mechanism involves monoamine oxidase inhibition, particularly MAO-A, resulting in increased serotonin and dopamine levels (Lee et al., 2005). This mechanism closely parallels conventional antidepressant approaches, suggesting *P. longum*'s potential as a natural alternative to synthetic MAO inhibitors. However, the clinical evidence for *P. longum* remains limited, with most studies conducted in preclinical models. *W. somnifera* demonstrates a more complex

mechanism involving GABAergic modulation, HPA axis regulation, and neurotrophic factor enhancement (Chandrasekhar et al., 2012). The plant's adaptogenic properties contribute to its antidepressant effects through stress response normalization and cortisol regulation. The extensive clinical evidence supporting *W. somnifera*'s efficacy, combined with its excellent safety profile, positions it as a well-validated botanical antidepressant. The phytochemical differences between the plants contribute to their distinct therapeutic profiles. *P. longum*'s high piperine content correlates with its potent MAO inhibitory activity, while *W. somnifera*'s withanolide composition supports its adaptogenic and anxiolytic properties (Mishra et al., 2000). These compositional differences suggest potential synergistic effects when used in combination, addressing multiple pathways involved in depression pathophysiology.

The clinical efficacy data consistently favors *W. somnifera*, with higher response rates and greater symptom improvement across multiple studies. This superiority likely reflects the more extensive research investment in *W. somnifera* and the development of standardized extraction protocols. The broader mechanism of action may also contribute to *W. somnifera*'s superior clinical outcomes, addressing both the neurochemical and stress-related components of depression. Safety considerations favor both plants over conventional antidepressants, with minimal adverse events and no significant drug interactions. However, *P. longum*'s MAO inhibitory activity requires consideration of dietary restrictions and potential interactions with other medications. *W. somnifera*'s excellent safety profile supports its use in various patient populations, including those with comorbid conditions. The standardization and dosage protocols reflect the maturity of research for each plant. *W. somnifera*'s well-established protocols, based on withanolide content, facilitate consistent clinical outcomes. *P. longum*'s standardization based on piperine content provides a foundation for therapeutic consistency, though further research is needed to optimize dosing protocols.

7. Conclusion

This comparative assessment demonstrates that both *P. longum* and *W. somnifera* possess significant antidepressant properties through distinct mechanisms of action. *W. somnifera* emerges as the more clinically validated option, with extensive evidence supporting its efficacy and safety in depression management. The plant's adaptogenic properties, combined with its excellent safety profile and established therapeutic protocols, make it a preferred choice for clinical application. *P. longum* shows promise as a natural antidepressant, particularly for its potent MAO inhibitory activity and neurotransmitter modulation effects. However, the limited clinical evidence and need for further standardization protocols limit its immediate clinical utility. The plant's unique mechanism of action suggests potential value in combination therapies or as an alternative for patients who do not respond to conventional treatments. The integration of both plants in comprehensive treatment approaches may offer enhanced therapeutic outcomes by addressing multiple pathways involved in depression pathophysiology. Future research should focus on combination studies, optimization of standardization protocols, and long-term safety assessments to fully realize the therapeutic potential of these valuable botanical resources.

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Tritiya Sansodhan Adhiniyam, 2011 Dated 31 Dec. 2011

Ref. No. SVNU/MISC/0203 -01

Date: 26/11/2023

IDENTIFICATION CERTIFICATE

This is to certify that, I have identified the plant specimen given by candidate Ms. RAGINEE KORI, student of Ph.D., in P.K. University, Shivpuri (M.P.). The plant details are as follows -

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Piperales

Family: Piperaceae

Genus: Piper

Species: P. longum

Botanical name: Piper longum

Local name: Long pepper

Herbarium Number BoT/H/07/33/06 is given by In-charge, Herbarium, Department of Botany, Faculty of Science, Swami Vivekanand University, Sagar (M.P.).

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Ref. No. SVNU/MISC/0204-02

Date: 26/11/2023

IDENTIFICATION CERTIFICATE

This is to certify that, I have identified the plant specimen given by candidate Ms. RAGINEE KORI, student of Ph.D., in P.K. University, Shivpuri (M.P.). The plant details are as follows -

Kingdom: Plantae
Division: Tracheophytes
Class: Magnoliopsida
Order: Solanales
Family: Solanaceae
Genus: Withania
Species: W. somnifera
Botanical name: Withania somnifera
Local name: Ashwagandha

Herbarium Number. BoT/H/05/14/21 is given by In-charge. Herbarium, Department of Botany, Faculty of Science, Swami Vivekanand University, Sagar (M.P.).

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