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ORIGINAL ARTICLE

PHYTOCHEMICAL SCREENING, TLC AND HPLC-BASED PROFILING OF BIOACTIVE COMPOUNDS IN *ZINGIBER OFFICINALE* RHIZOMES AND THEIR *IN VIVO* ANTI-INFLAMMATORY EFFECTS IN ANIMAL MODELS

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ABSTRACT : This paper will be analyzing the antioxidant and anti-inflammatory properties of *Zingiber officinale* rhizomes through a set of tests, which includes a series of *in vivo* experiments, phytochemical screening, HPLC and Thin Layer Chromatography. The screening methods using phytochemicals demonstrated a wide range of concentration of bioactive compounds with methanol and ethanol extracts showing the highest concentration of flavonoids, tannins, glycosides, phenolic compounds, and terpenoids, which have antioxidant and anti-inflammatory properties. TLC and HPLC studies proved the presence of the main compounds including gingerol among others, which are involved in most of the therapeutic effects of ginger. The HPLC indicates that methanol is the best solvent to use in extraction of gingerol. To investigate the *in vivo* anti-inflammatory effect of high-dose ginger extract (800 mg/kg), the carrageenan-induced paw edema model was used. The outcome revealed that the paw edema was reduced dose-dependently just like aspirin (200 mg/kg). These results may suggest that *Zingiber officinale* may react similarly to the well-known anti-inflammatory drugs that involve the inhibition of the COX-2 enzymes as well as the inhibition of the pro-inflammatory mediators. Overall, this paper suggests that *Zingiber officinale* rhizomes can be used as natural alternative to synthetic anti-inflammatory drugs with favorable safety history as a therapeutic option.

Key words : *Zingiber officinale*, TLC, HPLC, bioactive compounds, anti-inflammatory activity, carrageenan-induced paw edema.

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INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) rhizomes are ancient products of traditional medicine that has a variety of medicinal purposes. As a member of the family Zingiberaceae, ginger has popularized hefty anti-inflammatory, antioxidant, and anticancer effects, most of which have been linked to its high phytochemical content gingerols, shogaols and other biochemical compounds (Gupta and Sharma, 2014). These substances have been found effective in the treatment of a number of inflammatory diseases, including arthritis, osteoarthritis, and rheumatoid arthritis which can be attributed to the inhibition of pro-inflammatory enzymes and cytokines (Shahrajabian *et al,* 2019).

A multi-step immune system reaction to potentially dangerous substances, inflammation, and it is the focus of the pathogenesis of the majority of chronic diseases (Khatami, 2009). Although, they are successful, traditional nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently linked to such side effects as gastrointestinal irritation and cardiovascular risks, which requires finding safer and natural forms of these products. Plant-derived compounds that are gaining increasing popularity as possible therapeutic agents are bioactive compounds because of their safety profiles and their complex mechanism of action (Fiorino *et al*, 2021).

The phytochemical constituents in ginger are strongly correlated with its pharmacological action and can only be efficiently extracted *via* optimal solvents and detected by modern chromatographic and spectroscopic methods including: TLC, HPLC, UV-Visible and FTIR spectroscopy. The techniques will help to qualitatively and quantitatively describe major bioactive molecules that

contribute to the medicinal potential of ginger (Akullo *et al*, 2023).

Although, there is extensive traditional use, the scientific assessment of the *Zingiber officinale* extracts is important to ensure that the effectiveness and safety of *Zingiber officinale* extracts are tested and standardized through comprehensive scientific evaluation and phytochemical profiling, *in vitro* and *in vivo* pharmacological studies (Ali *et al*, 2008). The current research paper will serve to validate the plant material, determine the phytochemical composition of the sample with multiple solvents and state-of-the-art methods of analysis, and test the antioxidant and anti-inflammatory action of the material on a series of *in vitro* screening tests and a carrageenan-induced paw edema rat model. Moreover, acute toxicity studies are performed to evaluate the safety profile of ginger to support the possibility of its use as a natural anti-inflammatory agent (Ozkur *et al*, 2022).

This study adds to the growing literature indicating the therapeutic possibilities of *Zingiber officinale* and it offers a scientific rationale to the advancement of this particular substance as a safe and effective alternative to the traditional anti-inflammatory drugs.

MATERIALS AND METHODS

Plant Material collection and preparation

The *Zingiber officinale* (family: Zingiberaceae) rhizomes used in this study were freshly extracted in the month of October 2023 in one of the local gardens where Zingiberaceae is in full flower. The washing of the rhizomes with distilled water eliminated the soil and the debris, dried them in the shaded controlled conditions at room temperature, and finally ground them into a fine powder using a laboratory grinder to maintain the phytochemical integrity. The powdered material was kept in airtight containers to keep out moisture and light until extraction (Dineshbabu *et al*, 2023).

Extraction procedure

Approximately 500g of powdered rhizomes were extracted using a Soxhlet apparatus using a mixture of petroleum ether, chloroform, methanol, ethanol, and distilled water over a 24hr period. The solvents followed in the process were in descending sequences of polarity. Upon extraction, water bath was used to evaporate solvents at 50ºC in order to yield concentrated extracts. Extracts were kept in closed containers at room temperature to be used in additional analyses (AL-Ghudani and Hossain, 2015).

Phytochemical screening

Qualitative phytochemical screening was done to extract the potential bioactive components (Singh *et al*, 2022). Standard techniques were used to identify the following substances: alkaloids, carbs, glycosides, phytosterols, tannins, phenols, proteins, flavonoid, terpenoids, steroids and saponins. Key procedures included:

- Alkaloids: Dragendorff's test indicated by formation of an orange-red precipitate.
- Carbohydrates: Molisch's test producing a violet ring at the interface.
- Glycosides: Keller-Kiliani test showing a reddish-brown ring.
- Phytosterols: Salkowski test with reddish-brown interface on reaction with concentrated H, SO,, and chloroform.
- Fixed Oils & Fats: Spot test based on grease stain on filter paper.
- Tannins: Ferric chloride test producing blue-black or green coloration.
- Phenols: Ferric chloride test indicated by blue-green color development.
- Proteins: Biuret test producing violet or purple coloration.
- Flavonoids: Shinoda test indicated by red or pink pigment formation.
- Terpenoids: Salkowski test showing reddish-brown interface layers.
- Steroids: Liebermann-Burchard test showing greenish-blue color.
- Saponins: Foam test evidenced by sustained frothing.

These tests have been performed under the set procedures in order to validate the existence of compounds that add medicinal effects to the *Zingiber officinale* rhizomes.

Thin Layer Chromatography (TLC) profiling

Plate Preparation: Silica gel TLC plates precoated with a 0.25 mm layer were activated by heating at 110°C for 1 hour to remove moisture and enhance adsorption.

Sample Preparation: Methanol extracts were dried and then at the rate of 1 mg/mL. The extracts of each were 5 µL because in a drop, carefully dropped onto the TLC plate and 1 cm between the spots to prevent overlaps.

Mobile Phase: The mobile phase was a solvent system comprising of petroleum ether, ethyl acetate and methanol in the ratio of 8:2: 0.5 (v/v/v).

Chromatogram Development: A tank equipped with mobile phase solvent was loaded with the TLC plate in an upright position. The solvent was left to rise until it almost covered a plane of around 75 percent of the plate height, and then the plate was taken off and dried in air.

Visualization: The chromatogram thus prepared was viewed under ultra violet light at wavelengths 254 nm and 365 nm to identify compounds that absorb UV light or which have fluorescent properties. Also, the plate was sprayed by certain reagents to promote the spotting: iodine vapor in the general compounds, anisaldehyde reagent in flavonoids, and the Dragendorff reagent in alkaloids (Brolis *et al*, 1998).

Retention Factor (R_f) Calculation: The formula used to calculate the R_f value of each spot was:

$$Rf = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent front}}$$

Compound identification: To aid in the categorisation of bioactive compounds found in the extracts, the values of R_f, observed spot values, and the phytochemical standards were compared.

High Performance Liquid Chromatography (HPLC) analysis

High-performance liquid chromatography (HPLC) analysis was done in an LC-20A HPLC coupled with a UV-Visible detector set to 254 nm. A reverse-phase C18 HPLC column (250 mm × 4.6 mm, 5 mm) was utilized in the HPLC studies. The mobile phase consisted of 70% ethanol: 30% water (v/v) and 0.1% formic acid and sonicated to remove air. To analyze them, methanolic extracts were reconstituted to 10mg/mL unfiltered with a 0.45um filter, 20ul injected (1.0 mL/min) using the pump with the water bath at 0ºC and column temperature at 1mL. The temperature was 0ºC, 20ºC, the peaks were measured at 254 nm and the time zone and area of the peak was measured at 254 nm. The phytochemical peaks were determined by the use of time and literature. In case of phytochemical peaks, the peaks were assessed using literature and time (Ito, 1993).

***In vivo* anti-inflammatory activity in Animal Models**

Experimental Animals : We used adult Swiss albino mice (25-30 grams) purchased from a licensed breeder. The animals were kept in a controlled habitat with temperature and placed under "no experimental" circumstances. With a 12-hour light/dark cycle, the temperature was controlled at "22 ± 2°C" and the humidity at "50-60% relative humidity." They were given free access to water and rodent feed as needed (Moinet *et al*, 2021). The mice were kept for 7 days for acclimatisation. The Animal Ethics Committee approved all the tasks presented in the experiment (Approval No.: CCSEA/IAEC/JLS/22/10/24/025).

Plant Extract Preparation for In Vivo study : Fresh rhizomes of the plant were shade-dried and ground into a fine powder. This powdered material was extracted by maceration using ethanol or distilled water (the specific solvent used should be specified) for 48 hours with occasional stirring. Whatman No. 1 filter paper was used after the extract was filtered through muslin cloth. Under reduced pressure and a temperature of no more than 40°C, the concentration was carried out in a rotary evaporator. For future reference, the extract was further dried and kept at 4°C until needed (Urama *et al*, 2024).

Grouping and Treatment : Each of the five groups of six mice was given an equal number of mice at random. As a placebo, Group I received 10 mL/kg of distilled water orally. Substantially, 200 mg/kg of aspirin was given orally to Group II, the control group. Experimental groups III, IV, and V were given 400 mg/kg, 600 mg/kg, and 800 mg/kg of the plant extract orally, correspondingly (Pritchard *et al*, 2001).

Induction of Inflammation : Acute inflammation was induced in the animals one hour after treatment administration. To simulate acute inflammation, 0.05 mL of a 1% carrageenan solution was injected into the sub-plantar area of the left hind paw of each mouse (Anka *et al*, 2022).

Measurement of Paw Edema : Before the injection of carrageenan, the paw volume was first measured, and then at the 1, 2, 3, 4 and 5 hour marks after injection, the volume was measured again. A digital plethysmometer was used to ensure precise volume measurement (Sharma *et al*, 2004).

Evaluation of Anti-Inflammatory effect : The reduction in paw volume relative to the control group served as a quantitative measure of the effect of the test extract on inflammation (Chandra *et al*, 2012). The formula used for calculating the five percent inhibition of oedema was:

$$\% \text{ Inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

where, V_c is the mean increase in paw volume of the control group and V_t is the mean increase in paw volume of the test or standard group.

2.6.7. Statistical analysis : The data was represented by mean and standard error of the mean (SEM). Tukey post hoc test was applied to carry out statistically significant comparisons of the groups through one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered a sign of statistical significance (Lee *et al*, 2015).

RESULTS AND DISCUSSION

Phytochemical screening

Extracts from the rhizome of the *Zingiber officinale* plant were phytochemically screened and found to contain a variety of bioactive chemicals; the ethanol and methanol extracts contained the most components. The compounds that are most important are flavonoid, tannins, glycosides, phenolics and terpenoids. These compounds, which exist, show that the ginger rhizome still possesses a huge pharmacological potential since, it remains highly applicable in the treatment of various ailments as it always was the case.

The methanol and ethanol extracts showed the broadest spectrum of bioactive compounds, which are likely responsible for the medicinal properties of ginger. The presence of flavonoid and phenolic compounds is of particular interest because both of them are known to possess antioxidant and anti-inflammatory effects.

Thin Layer Chromatography (TLC) Profiling

Thin Layer Chromatography (TLC) was used to analyse *Zingiber officinale* extracts. The spots of different retention factors (Rf) were found to be different at UV light and they were assigned to different bioactive compounds that were predominantly flavonoid and terpenoid compounds. R_f of each extract was compared to the known phytochemical standards to ascertain the existence of these bioactive compounds.

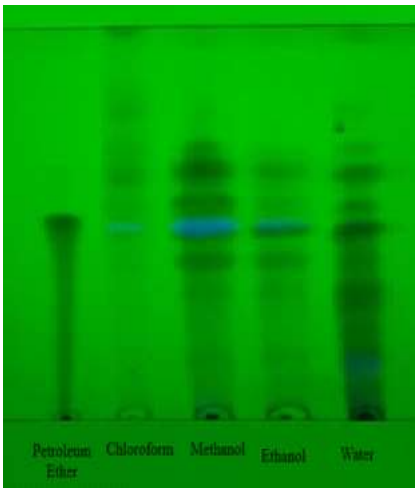

Fig. 1 : TLC Chromatogram of *Zingiber officinale* extracts.

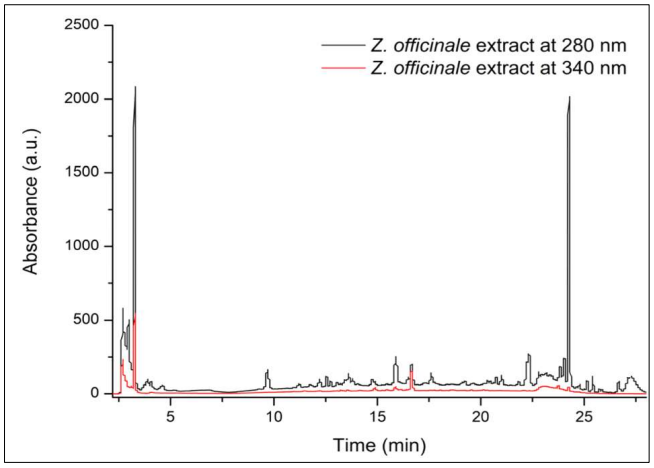

Fig. 2 : HPLC Chromatogram of *Zingiber officinale* extract.

Clear bands were seen in TLC chromatogram which revealed the presence of flavonoid and terpenoids in extracts. The values of the R_f obtained were comparable

Table 1 : Qualitative Phytochemical screening of *Zingiber officinale* Rhizomes extracts.

Phytochemical	Petroleum Ether	Chloroform	Methanol	Ethanol	Water
Alkaloids	-	-	+	+	-
Flavonoids	-	-	+	+	+
Tannins	-	-	+	+	+
Saponins	-	-	-	-	+
Glycosides	-	-	+	+	+
Terpenoids	+	+	+	+	-
Phenolic Compounds	-	+	+	+	+

Table 2 : TLC Data of *Zingiber officinale* Rhizomes extracts.

Extract Type	Distance Travelled by Solvent (cm)	Distance Travelled by Solute (cm)	Rf Value
Petroleum Ether	18	8.3	0.46
Chloroform	18	8.8	0.49
Methanol	18	8.5	0.47
Ethanol	18	8.6	0.48
Water	18	8.1	0.45

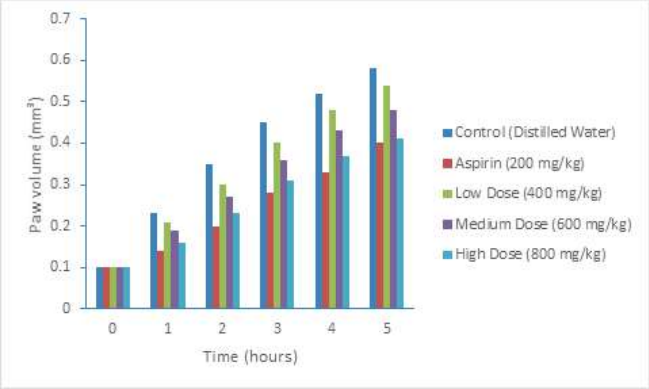

Fig. 3 : Time-Dependent effect of *Zingiber officinale* extract on Carrageenan-Induced Paw edema.

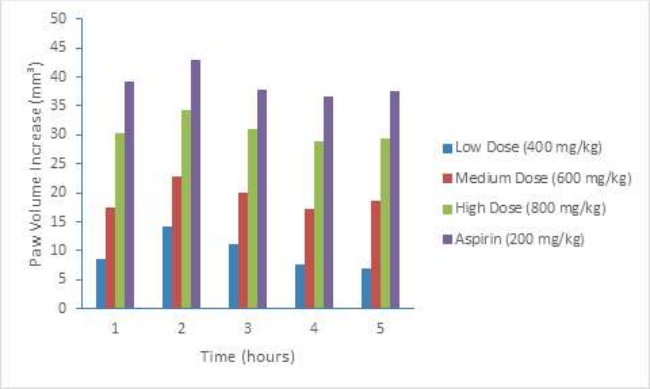

Fig. 4 : Percentage Inhibition of Paw edema by *Zingiber officinale* Rhizomes extract and aspirin.

Table 3 : HPLC Retention Time and Peak Area Data for *Zingiber officinale* extract.

Peak Number	Retention Time (min)	Absorbance at 280 nm (a.u.)	Peak Area at 280 nm (a.u.)
1	5.0	2000	15000
2	8.0	300	2000
3	12.0	100	1200
4	15.0	250	1800
5	18.0	150	1100
6	20.0	50	500

Table 4 : Paw Edema measurement at different Time points.

Time (hours)	Control (Distilled Water)	Aspirin (200 mg/kg)	Low Dose (400 mg/kg)	Medium Dose (600 mg/kg)	High Dose (800 mg/kg)
0	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
1	0.23 ± 0.02	0.14 ± 0.03	0.21 ± 0.02	0.19 ± 0.02	0.16 ± 0.02
2	0.35 ± 0.04	0.20 ± 0.03	0.30 ± 0.03	0.27 ± 0.02	0.23 ± 0.02
3	0.45 ± 0.03	0.28 ± 0.03	0.40 ± 0.04	0.36 ± 0.03	0.31 ± 0.02
4	0.52 ± 0.05	0.33 ± 0.03	0.48 ± 0.04	0.43 ± 0.03	0.37 ± 0.03
5	0.58 ± 0.06	0.40 ± 0.04	0.54 ± 0.05	0.48 ± 0.04	0.41 ± 0.03

Table 5 : Percentage Inhibition of Paw Edema.

Time (hours)	Low Dose (400 mg/kg)	Medium Dose (600 mg/kg)	High Dose (800 mg/kg)	Aspirin (200 mg/kg)
1	8.7%	17.4%	30.4%	39.1%
2	14.3%	22.9%	34.3%	42.9%
3	11.1%	20.0%	31.1%	37.8%
4	7.7%	17.3%	28.8%	36.5%
5	6.9%	18.7%	29.3%	37.5%

with known standards of these compounds.

High-Performance Liquid Chromatography (HPLC) analysis

On HPLC, the rhizome extracts of *Zingiber officinale* were studied using unemployed reverse-phase C18 column. The chromatogram presented several peaks, and the major ones were those of gingerols, one of the major compounds that contribute to anti-inflammatory and antioxidant effects of ginger. Methanol extract had the highest concentration of these compounds.

In vivo anti-inflammatory activity

The carrageenan-induced paw edema model was used to assess the anti-inflammatory efficacy of *Zingiber officinale in vivo*. All treatment groups demonstrated a notable decrease in paw volume that was dose-dependent. Similar to the recognized anti-inflammatory medicine aspirin (200 mg/kg), the high-dose (800 mg/kg) group showed the most substantial reduction in paw edema.

DISCUSSION

Phytochemical screening of *Zingiber officinale* rhizome extracts showed a high composition of bioactive

compounds especially in the methanol and ethanol extracts which had the widest spectrum of compounds. Some of the extracts contained flavonoids, tannin, glycosides, phenolics and terpenoids all which have been reported to possess high antioxidant and anti-inflammatory properties. These extracts reveal the medicinal nature of ginger since it had been applied in the treatment of various disorders in the past. Table 1 provides a summary of the compounds that are identified in the different solvent extracts. The concentration of the bioactive compounds, including flavonoids, tannins, glycosides, phenolic compounds and the petroleum ether extract was also dominated by terpenoids, as it can be seen in the table, whereas, the concentration of saponins and phenolic compounds was high in the water extract. It is possible to assume that the therapeutic importance of ginger is due to the availability of the methanol and ethanol extracts because both extracts have diverse bioactive compounds.

Thin Layer Chromatography (TLC) was performed to determine the bioactive compounds present in the extracts of the rhizome and the results are summarised in Table 2 which also contains the distance that the solute and solvent front moved. The R_f values of the extracts were compared to the phytochemical standards of flavonoid and terpenoid compounds which are known to be present in the methanol and ethanol extracts. Fig. 1 shows the TLC chromatogram, where resolutions of these compounds were viewed with the help of the UV light. The ability of methanol and ethanol in extracting these useful bioactive compounds in ginger that exhibits antioxidant and anti-inflammatory properties was shown graphically by presenting the chromatogram. The presence of flavonoids and terpenoids in these extracts is indicative of this fact as well and it can be used to denote that they contribute to the therapeutic properties of ginger.

The bioactive compounds in the extracts of rhizomes were further analysed using High-Performance Liquid Chromatography (HPLC). Table 3 shows the retention time and the largest area of the HPLC analysis with the primary peak referring to gingerol as the largest bioactive agent of anti-inflammatory and anti-oxidant effects. The highest concentration of gingerols was found in Methanol extract due to the highest peak areas and the absorbance at 280 nm. The chromatogram of the HPLC in Fig. 2 illustrates that the steep peak of the HPLC chromatogram of around 5 minutes depicts the presence of gingerol and the other lower peaks depict the presence of other bioactive compounds. The HPLC results confirm the hypothesis that the bioactive ingredient of gingerol is the main active component in the *Zingiber officinale* and that methanol will be the optimal solvent to use.

The *in vivo* anti-inflammatory activity of *Zingiber officinale* was determined with the assistance of the carrageenan induced paw edema model that is one of the most commonly used tests to assess acute inflammation. Table 4 shows the paw edema of the different treatment groups of control group, aspirin group (200 mg/kg) and the different doses of the ginger extract (400 mg/kg, 600 mg/kg, and 800 mg/kg). The outcomes indicated a dose-dependent significant decrease in the paw volume in all the treatment groups. The most remarkable edema reduction was observed in the high dose (800 mg/kg) of the test, and it was similar to aspirin (200 mg/kg), which is a known anti-inflammatory medication. The effect of ginger extract on paw edema with time is visually represented in Fig. 3. The largest decrease in paw volume was observed in the high-dose group in all time points which supported the strong anti-inflammatory action of ginger.

Table 5 and Fig. 4 present the percentage paw edema inhibition of all groups. Percent inhibition was determined by dividing the increase in the paw volume of treatment groups by the average increase in the control group. The high dose (800 mg/kg) group (37.5% at 5 hours) had the highest percentage inhibition of the paw edema, just like the aspirin group. This also verifies the hypothesis that active compounds in *Zingiber officinale* such as gingerol are included in the anti-inflammatory activity of the extract, which is demonstrated to interact with the inflammatory pathways. These findings suggest that ginger may possibly act on the same path as aspirin, most likely by inhibiting COX-2 enzymes and the production of pro-inflammatory mediators.

In conclusion, the assimilation of the phytochemical examination, TLC, HPLC and the *in vivo* anti-inflammatory information are quite promising signs of the medicinal *Zingiber officinale* rhizomes. The existence of bioactive compounds such as gingerol, flavonoid and phenolic compounds of the waste in the methanol and ethanol extracts in the assumption that the compounds are the ones that drive the therapeutic effect. The *in vivo* studies particularly the carrageenan induced paw edema reveal that *Zingiber officinale* contains potent anti-inflammatory properties and it has equal or better effects compared to aspirin. These results imply that ginger can be considered as a quality natural substitute of anti-inflammatory medications in the pharmaceutical market, and the fact that ginger is a well-known ingredient in terms of safety is a bonus.

CONCLUSION

Concluding, this paper documents the excellent research findings regarding the strong anti-inflammatory and antioxidant effects of the *Zingiber officinale* rhizomes. Phytochemical screening, Thin Layer Chromatography (TLC) and High-performance liquid chromatography (HPLC) screening have shown a broad spectrum of bioactive compounds particularly flavonoids, phenolic compounds and gingerol which are the attributable cause of the therapeutic effect of the plant. They found that the extracts that were the most active were the ethanol extracts and the methanol extracts that helped support their contribution to *Zingiber officinale* medicinal properties. Anti-inflammatory effect in in vivo condition was determined by the carrageenan induced paw edema model which showed a dose effect of significant degree (800mg/kg) that corresponded to aspirin that is a standard anti-inflammatory agent. This corroborates the hypothesis that *Zingiber officinale* can have its anti-inflammatory effects by inhibiting the COX-2 enzymes and decreasing the pro-inflammatory mediators. *Zingiber officinale* rhizomes have a potential to serve as a natural substitute of conventional therapeutic approaches to the inflammation and oxidative stress-related disorders due to its excellent therapeutic potential and a good safety profile. More clinical research is justified to establish the entire range of its medical advantages in human beings.

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