Multimodal protection against oxidative stress mediated damage in cancer: An attempt to explore combitorial drug therapy with Non-Steroidal Anti-Inflammatory Drug (NSAID) and neutraceutical as its component

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By

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Date: (**Mahendra Pratap Singh)**

Place:

LIST OF ABBREVIATIONS

Abbreviations	Definition
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
MAPK	Mitogen-activated protein kinase
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
mAbs	Monoclonal antibodies
TIMPs	Tissue inhibitors of metalloproteinases
IHC	Immunohistochemical
CT	Computed tomography
MRI	Magnetic resonance imaging
IARC	International agency for research on cancer
WHO	World health organization
Ki	Inhibition constant
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
GLOBOCAN	Global cancer observatory
NSAID	Nonsteroidal anti-inflammatory drug
ELISA	Enzyme-linked immunosorbent assay
RCSB	Research Collaboratory for Structural Bioinformatics
TPSA	Total polar surface area
LD	Lethal Dose
ROS	Reactive oxygen species
ARE	Activates antioxidant response element
IC	Inhibitory concentration
nm	Nano meter
ml	Milli liter
µg	Micro gram
µM	Micro molar
µl	Micro liter

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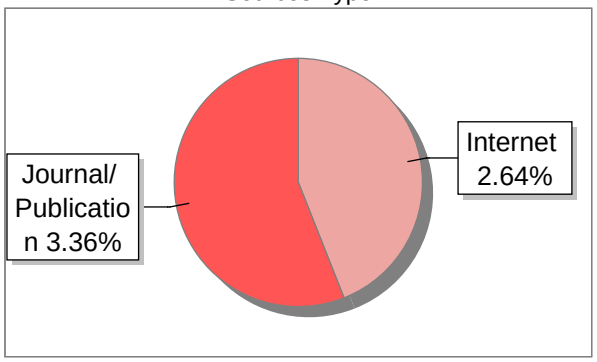

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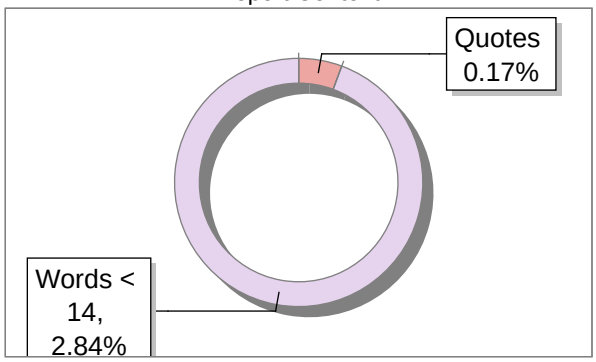

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ABSTRACT

Cancer ranks as the world's second most common cause of death, is characterized by uncontrolled cell proliferation and metastasis alteration in genetic level that activate oncogenes and deactivate tumor suppressor genes. Matrix metalloproteinase-9 (MMP-9), or gelatinase B, contributes significantly to extracellular matrix degradation and the remodeling of tissues, angiogenesis, and tumor microenvironment formation, with its overexpression documented in nearly all cancer types. Targeting MMP-9 has thus emerged as a promising therapeutic strategy. In this study, we employed an in-silico and in-vitro approach to investigate potential MMP-9 inhibitors from two distinct chemical classes—natural flavonoids and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)—both individually and in synergistic combinations. ADMET and bioactivity profiling of selected flavonoids identified luteolin and quercetin as the most promising candidates, with molecular docking revealing luteolin as the strongest individual inhibitor. Combination docking identified quercetin–genistein and luteolin–genistein pairs with binding energies of −15.48 and −15.31 kcal/mol, respectively, surpassing the affinities of individual ligands. Similarly, among NSAIDs, oxaprozin and piroxicam demonstrated the highest individual binding affinities, with their combination yielding a binding energy of −12.98 kcal/mol. Further cross-class docking of the top candidates luteolin and piroxicam showed substantial inhibitory potential. Experimental validation using DPPH scavenging and MTT assays confirmed the combination's strong antioxidant capacity and cytotoxicity, with favourable IC_{50} values, suggesting its efficacy in combating oxidative stress and MMP-9–mediated cancer progression. Overall, our findings highlight the potential of synergistic flavonoid–NSAID combinations as a novel strategy for MMP-9 inhibition, warranting further in vitro and in vivo evaluation for high-efficacy cancer therapy.

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CHAPTER – 1

INTRODUCTION

1.1. Cancer and Its Pathologies

Cancer is a brought disease, encompassing a vast spectrum of disorders characterized by abnormal and uncontrolled cell growth, with the potential to invade adjacent tissues and metastasize to distant organs. Globally, it is a leading cause of mortality, accounting for millions of deaths annually, and its incidence is expected to rise in the coming decades due to aging populations, lifestyle factors, and environmental exposures (Sung et al., 2021). At the molecular level, cancer develops through a multistep process involving the accumulation of genetic mutations and epigenetic alterations (Hanahan & Weinberg, 2011) This process is fueled by genomic instability, which facilitates the acquisition of additional mutations that drive tumor progression. In cancer research, the concept known as the "hallmarks of cancer" serves as a core model for understanding the disease, which describe the common capabilities acquired by most malignant cells, including ongoing proliferative cues, suppression-evading mechanisms, induction of angiogenesis, invasion activation and metastasis, reprogramming of energy metabolism, and evasion of immune destruction (Hanahan, 2022). These hallmarks are not isolated traits but interact dynamically within the tumor and its microenvironment, making cancer a highly adaptive and resilient disease.

The tumor microenvironment is crucial in driving cancer initiation and progression. It comprises a heterogeneous mixture of cancer cells, fibroblasts, and the extracellular matrix, all of which engage in complex signaling networks (Quail & Joyce, 2013). Contextually, these interactions have the potential to suppress tumors or enhance malignant advancement. Chronic inflammation within the TME, often driven by infection, autoimmune disorders, or environmental factors, acts as a tumor-promoting force by generating reactive oxygen and nitrogen species, enhancing DNA damage, and stimulating angiogenesis and tissue remodeling (Greten & Grivennikov, 2019). Moreover, cancer cells can reprogram surrounding stromal cells to create an immunosuppressive, niche that enables evasion of

immune system surveillance. Tumor-associated macrophages, for instance, often adopt a pro-tumoral phenotype that supports angiogenesis, extracellular matrix degradation, and metastasis (Mantovani et al., 2017). Metabolic reprogramming, the Warburg effect — A state in which cancer cells preferentially depend on aerobic glycolysis — provides both energy and biologically synthesised precursors required for accelerated proliferation, while also influencing the epigenetic landscape and gene expression patterns of tumor cells (Pavlova & Thompson, 2016). These adaptive features make targeting the TME for cancer therapy.

Metastasis remains the most lethal attribute of malignant tumors, accounting for the vast majority of cancer-related deaths (Steeg, 2016). The metastatic cascade consists of multiple stages, including local invasion of nearby tissues, entry into blood or lymphatic vessels (intravasation), survival within the circulation, exit into distant tissues (extravasation), and eventual colonization of secondary sites. Each of these steps requires distinct molecular and cellular adaptations, including loss of cell–cell adhesion through downregulation of E-cadherin, acquisition of motility through epithelial–mesenchymal transition (EMT), and resistance for anoikis, triggered by detachment from the ECM (Lambert et al., 2017). Organ-specific metastasis patterns reflect both anatomical factors, like vascular drainage routes. Like breast cancer often metastasizes to bone, while colorectal cancer frequently spreads to the liver. Understanding these organotropisms is essential for developing strategies to prevent or treat metastatic disease. Despite advances in surgery, radiotherapy, chemotherapy, and targeted therapy, metastasis remains a formidable clinical challenge due to its heterogeneity and with therapy-resistant tumor cell subpopulations.

Cancer is also a systemic disease, with widespread effects beyond the primary tumor site. Tumor-induced cachexia, a multifactorial syndrome Characterized by unintended weight loss and degeneration of muscle tissue and metabolic disturbances, significantly reduces patient quality of life and response to therapy (Fearon et al., 2011). This syndrome is driven by systemic inflammation, altered metabolism, and the factor that derive tumor such as pro-inflammatory cytokines and proteolysis-inducing factors. Anaemia, immunosuppression, and

thromboembolic events are other frequent systemic manifestations of malignancy, reflecting the profound physiological disruptions caused by tumor growth. Addressing these systemic effects is critical in comprehensive cancer care, as supportive therapies can improve both survival and life style quality. Therefore, cancer pathologies are the result of intricate and dynamic interactions between genetic mutations, epigenetic reprogramming, metabolic changes, and microenvironmental influences. The hallmarks of cancer provide a fundamental framework for the study of these processes, while advances in molecular biology, pathology, and systems medicine continue to refine our knowledge. Future progress will depend on integrating these insights into early detection strategies, targeted interventions, and approaches that address both tumor-intrinsic and systemic aspects of the disease. By doing so, oncology can move closer to the goal of transforming cancer into a manageable chronic condition or achieving durable cures for an increasing number of patients.

1.2. Matrix metalloproteinases (MMPs) and Cancer

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a main function in extracellular matrix remodeling, a process that is critical for both physiological tissue homeostasis and pathological conditions such as cancer. In normal tissues, MMP activity is tightly regulated at the transcriptional level, by proenzyme activation, and through inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Nagase et al., 2006). This process happens through the breakdown of ECM components, which facilitates cancer cell invasion, migration, and metastasis, and through the release of ECM-associated growth factors that upregulate angiogenesis (Egeblad & Werb, 2002).

MMPs have been involve in modulating the tumor microenvironment by influencing immune cell infiltration, inflammation, and signaling cascades that elicit tumor survival. As per earlier investigation reported that oxidative stress—a hallmark of many cancers—can modulate MMP expression via redox-sensitive transcription factors such as NF-κB and AP-1, thereby creating a feedback loop where MMP activity exacerbates oxidative damage, and oxidative stress further amplifies MMP expression (Karin & Greten, 2005). This correlation between

oxidative stress and MMP activation not only accelerates tumor progression but also confers resistance to apoptosis, making MMPs potential therapeutic targets.

The MMPs and oxidative stress relationship in cancer is multifaceted. Free oxygen species generated through mitochondrial dysfunction, oncogene activation, or inflammatory processes, can directly activate latent proMMPs via oxidation of cysteine residues in their propeptide domains (Rajagopalan et al., 1996). Furthermore, ROS-mediated activation of mitogen-activated protein kinases (MAPKs) enhances MMP gene transcription, leading to increased proteolytic activity in the tumor microenvironment. In turn, excessive MMP activity promotes ECM breakdown, which releases bioactive fragments known as matrikines that can further initiate oxidants production like ROS by both cancer and stromal cells (Overall & Kleifeld, 2006). This bidirectional amplification contributes to tumor invasion, metastatic niche formation, and angiogenesis, all of which are essential for malignant progression. The elevated circulating levels of certain MMPs correlate with tumor aggressiveness and poor prognosis in number of cancers, such as breast, colorectal, lung, and pancreatic carcinomas (Kessenbrock et al., 2010). Importantly, the oxidative stress–MMP have been connected to therapy resistance, where chemotherapeutic agents inadvertently enhance ROS generation, triggering compensatory MMP upregulation that facilitates tumor relapse. These findings underscore the need to understand MMP regulation in the oxidative context for the development of more effective anti-cancer strategies.

Within the MMP family, MMP-9 (gelatinase B) has been widely investigated due to its significant involvement in cancer progression and its responsiveness to oxidative regulation. MMP9 specifically degrades collagen type IV, a major structural constituent of basement membranes, that support tumor cell intravasation and extravasation during metastasis (Vu & Werb, 2000). Elevated MMP9 expression has been identified in a wide range of malignancies, including breast, prostate, gastric, and glioblastomas, often correlating with advanced disease stage and poor patient survival (Deryugina & Quigley, 2006).

Oxidative stress plays a important role in MMP-9 activation; ROS-mediated pathways activate transcription factors like NF-κB, which in turn upregulate MMP-

9 transcription (Bond et al., 1998). Additionally, ROS can directly cleave and activate pro-MMP-9, amplifying its proteolytic capacity in the tumor milieu. This ROS–MMP9 synergy promotes not only ECM degradation but also enhancing angiogenesis and supporting tumor expansion. MMP-9 also shown to modulate immune surveillance by regulating cytokine and chemokine availability, thereby influencing tumor-associated inflammation (Parks et al., 2004). Considering its multifactorial role, MMP-9 is also actively examine as a biomarker for cancer diagnosis and prognosis, along with a therapeutic target. Inhibiting MMP-9 activity—either directly through small molecule inhibitors or indirectly by targeting upstream oxidative pathways—has been shown significant preclinical results, though applying this clinically is still difficult because of the complexity of MMP regulation in vivo.

1.3. MMP-9 and its inhibitors against cancers

Matrix metalloproteinase-9 (MMP-9), a member of the gelatinase subgroup of MMPs, plays a crucial function in extracellular matrix (ECM) degradation, facilitating processes such as tumor invasion, angiogenesis, and metastasis. Overexpression of MMP-9 has been identified in a wide range of malignancies, as in breast, gastric, pancreatic, and lung cancers, where its activity linked with poor prognosis and advanced tumor stage (Vandooren et al., 2013); (Gialeli et al., 2011)).

The proteolytic activity of MMP-9 not only enables the breaking of ECM constituents, but also regulates the bioavailability of growth factor like VEGF, thereby promoting angiogenesis and cancer cell survival (Jabłońska-Trypuć et al., 2016). MMP-9 is also implicated in oxidative stress–driven tumorigenesis, as reactive oxygen species (ROS) can upregulate MMP-9 transcription through redox-sensitive transcription factors namely NF-κB and AP-1 (Siwik et al., 2001). In tumor microenvironment, oxidative stress triggers a feed-forward mechanism where elevated MMP-9 levels enhance inflammation, further driving ROS production and cellular damage. Given its multifaceted role in cancer progression, MMP-9 is considered a critical biomarker and therapeutic target for both diagnostic and interventional strategies.

Most of the natural compounds may acts as inhibitors of MMP-9 because of their relatively low toxicity and ability to modulate multiple signaling pathways. Polyphenols such as epigallocatechin gallate (EGCG) from green tea and curcumin from turmeric reduce MMP-9 expression by interfering with MAPK and other signals (Annabi et al., 2002); (Aggarwal & Harikumar, 2009).

Flavonoids like quercetin and luteolin similarly downregulate MMP-9 activity and suppress tumor cell migration in vitro and in vivo (Liu et al., 2015). These natural compounds function via multiple pathways, such as suppressing transcription, blocking pro-MMP-9 activation, and neutralizing ROS, thereby mitigating oxidative stress–driven MMP-9 upregulation. While promising, the clinical translation of natural MMP-9 inhibitors faces limitations such as poor bioavailability, rapid metabolism, and inconsistent potency in human studies. Consequently, their therapeutic potential is often considered complementary to conventional anticancer treatments rather than as standalone interventions.

In addition to natural molecules, non-steroidal anti-inflammatory drugs (NSAIDs) have emerged as pharmacological inhibitors of MMP-9 with significant implications in oncology. NSAIDs, including aspirin, indomethacin, and celecoxib, have been reported to suppress MMP-9 expression by inhibiting COX-2–mediated prostaglandin E2 (PGE2) production, that downregulates MMP-9 transcription (Tsujii et al., 1998); Hwang et al., 2006). Selective COX-2 inhibitors such as celecoxib have demonstrated additional MMP-9 inhibitory effects independent of COX-2 blockade, including direct interference with Akt and ERK signaling cascades (Krysan et al., 2004). In preclinical models, NSAID treatment are connected with reduced tumor invasion, angiogenesis, and metastasis, correlating with decreased MMP-9 levels in both tumor tissue and serum (Jung et al., 2010). These findings underscore the potential of NSAID-based strategies as adjunctive therapies targeting MMP-9, particularly in cancers characterized by high MMP-9 activity. However, the risks of gastrointestinal and cardiovascular side effects necessitate careful dosing and patient selection in clinical applications.

Combination strategies that pair bioactive natural compounds with non-steroidal anti-inflammatory drugs (NSAIDs) are gaining attention as a means to suppress

matrix metalloproteinase-9 (MMP-9)–driven invasion and metastasis while potentially lowering doses and side effects of each agent. Natural products such as epigallocatechin-3-gallate (EGCG), curcumin, and various polyphenols downregulate MMP-9 transcription and activity through inhibition of NF-κB, AP-1 or MAPK signaling, and by Detoxifying free radical oxygen that otherwise induce MMP expression ((Annabi et al., 2002); Aggarwal & Harikumar, 2009). NSAIDs, particularly selective COX-2 inhibitors like celecoxib, reduce prostaglandin E_2 (PGE_2) signaling that feeds NF-κB–dependent MMP-9 expression and can also affect Akt/ERK pathways in a COX-independent manner (Tsujii et al., 1998; Krysan et al., 2004). Preclinical studies show that co-treatment with EGCG and celecoxib produces synergistic reductions in tumor cell viability, VEGF release and MMP family activity, indicating additive or synergistic blockade of both inflammatory (COX-2/PGE_2) and redox/transcriptional mechanisms that drive MMP-9 (Noda et al. and Zhang et al.; PC-9 and Colo357 cell studies). Such dual targeting can blunt ECM degradation, restrict angiogenesis, and reduce invasive phenotypes are more effective (Khan et al.;)

Mechanistically, combinations capitalize on complementary actions: natural compounds often act upstream by lowering oxidative stress and inhibiting transcription factors that induce MMP-9, whereas NSAIDs suppress prostanoid-mediated pro-MMP signaling and downstream kinase cascades. For example, curcumin reduces MMP-9 via AMPK activation and NF-κB inhibition in colon cancer models, and when paired with agents that suppress COX-2 signaling the net effect on MMP-9 expression and invasion is amplified (curcumin studies). EGCG similarly downregulates MMP-9 and synergizes with celecoxib to enhance apoptosis and reduce invasiveness in lung and pancreatic cancer cell lines (EGCG + celecoxib). Importantly, other combined treatment have been examined in diverse tumor types (breast, colon, pancreatic, lung) and reported consistent trends: decreased MMP-9 expression/activity, reduced migration/invasion in vitro, and attenuated tumor growth or metastasis in vivo, supporting the translational rationale for combined nutraceutical–NSAID therapy.

Though analysis of natural compounds and NSAIDs for reducing the expression was investigated previously but studies on structural inhibition of MMP-9 by these compounds are limited.

1.4. Molecular Docking

Molecular docking is a widely used computational approach in structure-based drug design to predict the preferred orientation of a ligand when bound to its target protein, thereby estimating binding affinity and interaction modes. Molecular docking method has significant role in identifying potential inhibitors against specific targets such as matrix metalloproteinase-9 (MMP-9), which is implicated in tumor invasion, metastasis, and angiogenesis (Vihinen et al., 2005). Docking studies typically involve the preparation of the protein structure, retrieval or modeling of ligands, and applying an algorithm such as Autodock or Glide to predict ligand–protein interactions ((Morris et al., 2009); (Friesner et al., 2004)). Computational docking accelerates drug discovery by allowing in silico screening of large compound libraries, reducing time and cost before wet-lab testing. For MMP-9 inhibitors, both natural compounds and non-steroidal anti-inflammatory drugs (NSAIDs) have been assessed for binding potential, supporting their therapeutic relevance in oncology (Rashid et al., 2023).

1.5. MTT Assay

The MTT assay is a colorimetric method widely used to evaluate cell viability, proliferation, and cytotoxicity of compounds in cancer research. Its principle is based on the reduction of yellow tetrazolium salt (MTT) to insoluble purple formazan crystals by mitochondrial dehydrogenases in metabolically active cells ((Mosmann, 1983). In the context of MMP-9 inhibitor research, the MTT assay provides crucial in vitro data on the anti-proliferative effects of natural compounds, NSAIDs, or their combinations on cancer cell lines that exhibit elevated MMP-9 activity (Liang et al., 2007); (Li et al., 2023). The assay is typically performed by treating cancer cells culture at different concentrations of the test compound, incubating for a defined period, adding MTT reagent, and solubilizing the formed crystals before absorbance measurement at 570 nm. Reduction in cell viability

correlates with compound potency, and IC_{50} values are calculated. MTT results are often complemented assays to establish a broader picture of anti-metastatic potential, particularly when MMP-9 downregulation is targeted (Shen et al., 2012).

1.6. DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a fast assay, simple method to evaluate the antioxidant capacity of natural and synthetic compounds. The DPPH display a deep violet colour, which fades upon reduction by an antioxidant (Blois, 1958). Absorbance decrease at 517 nm quantitatively reflects radical scavenging activity. In MMP-9-related cancer studies, DPPH assays are used to find test compounds with antioxidant properties that can mitigate oxidative stress–mediated upregulation of MMP-9 expression, a key process in tumor progression (Wang et al., 2016; (Kessenbrock et al., 2010). Antioxidants from plant sources, such as flavonoids and polyphenols, have been shown to exert dual actions by scavenging ROS and directly inhibiting MMP-9 activity (Chou et al., 2010). NSAIDs with antioxidant-like properties, when combined with natural compounds, can offer synergistic effects in suppressing oxidative stress and MMP-9–driven metastatic pathways. The DPPH assay thus serves as an initial screening step to select candidates for further docking studies, cellular assays, and in vivo testing in cancer models (Ryou et al., 2011).

AIM

The aim of this study is to identify promising combinations of a natural molecule and an NSAID that synergistically inhibit MMP-9 activity by structural inhibition, specifically tailored to counter **oxidative stress–mediated upregulation of MMP-9** in cancer. By focusing on agents with complementary mechanisms—natural antioxidants and NSAID-mediated anti-inflammatory action—this research seeks to develop a targeted, dual-action therapeutic strategy to mitigate MMP-9–driven tumor progression under oxidative conditions.

OBJECTIVES

The objective of this study is to explore the combination therapy of NSAID and nutraceuticals in mediating MMP9 inhibition against cancer.

1. Molecular modelling of MMP-9 and Protein-drug interaction in-silico studies of nutraceutical combinations for MMP-9 inhibition

2. Screening of NSAID and plant nutraceutical combinations from medicinal plants to identify potential combination drug therapy against cancer.

3. Exploration of bioactivity of NSAID and nutraceuticals individually and in combination (MTT assays, antioxidant assay, ROS etc.) on cancer cell lines.

CHAPTER – 2

REVIEW OF LITERATURE

2.1 Background on Matrix Metalloproteinase (MMPs)

The Matrix Metalloproteinase (MMP) belongs to the zinc-dependent proteolytic enzyme, extensively studied since 1962, covering an enzyme in the mammalian uterus that degrades collagen in various animal and tissue models (Woessner, 1962). MMPs have been researched across disciplines like biochemistry, cell biology, pathology, immunology, physiology, and computational biology, focusing on diseases like arthritis, cancer, periodontal diseases, and cardiovascular diseases. In the late 1980s, additional MMPs were discovered and given the name MMPs (Okada et al., 1990). The MMP family has 25 members (Table 1). The family classification is based on sequence homology and substrate characteristics into collagenases, gelatinases, matrilysins, stromelysins, and membrane-type MMPs (Iyer et al., 2012)**.** These all are capable of degrading constituents of the ECM including collagen, fibronectin, laminin, and proteoglycan protein core (Cabral-Pacheco et al., 2020).

They are accountable for the deterioration and modification of the proteins that form the ECM. They have a proteolytic activity that have a significant role in different pathological and physiological processes, like as tissue remodeling, organ development, control of inflammatory functions, and cancer progression. The different classes of MMPs perform different functions, such as collagenase mediates the degeneration of triple-helical fibrillar collagen. Gelatinases are important in various physiological and cellular processes like wound healing, cell migration, and angiogenesis. Stromelysins have the potential to degrade laminin, fibronectin, gelatin, and collagen. Matrilysinsdegrade components of ECM. MT-MMPs are cell surface active enzymes and have collagenolytic and proteolytic activity towards ECM components. All MMPs have a protease domain and a conserved sequence HEXGHXXGXXHS/T with three histidine residues making a complex with a catalytic Zn atom and a regulatory conserved sequence domain PRCGXPD important for binding of cysteine to the Zn at active site found in the protease domain of MMPs (Fig.1) (Nagase et al., 2006).

Table 1: - Classification of MMPs

MMPs	Cancer progression stages	Action	Results
MMP-2, MMP-9, MT1-MMP	Invasion	Proteolytic	ECM macromolecules degradation
MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-11, MMP-19	Proliferation	Insulin-like growth factor (IGF)-binding protein cleavage	Proliferation
MMP-3, MMP-7		Release the cell membrane precursors of several growth factors, (HB-EGF, TGF-α, and amphiregulin)	Proliferation
MMP-9, MMP-2, MMP-14		TGF-β proteolytic activation	Proliferation
MMP-7		cleavage of HB-EGF	Proliferation
MMP-7	Apoptosis	Fas ligand cleavage	Resistance to apoptosis and chemoresistance to the cancer cells
MMP-2, MMP-9, MMP-14, MMP-1, MMP-7	Angiogenesis and Vasculogenesis	Extracellular constituents' degradation, such as collagen type IV, XVIII by secret of VEGF and basic fibroblast growth factor (bFGF)	Angiogenesis regulation
MMP-2	Cell adhesion, migration, and	Degradation of ECM molecule causes the generation of cryptic peptides	Facilitates cancer cell migration

MMP-2, MMP-3, MMP-9, MMP-13, MMP14	epithelial to mesenchymal transition	Excessive expression associated with epithelial-to-mesenchymal transition (EMT)	Morphological transition and migration
MMP-1, MMP-7		Cleavage of E-cadherin	Disrupted cell adhesion and induction of EMT causes morphological transition
MMP-28		Proteolytic activation of TGF-β	EMT inducer
MMP-9	Immune surveillance	Shed interleukin-2 receptor-α by the cell surface of T-lymphocytes	Suppressing proliferation
MMP-9, MMP-2, MMP-14		TGF-β release	Suppressor of T-lymphocyte reaction
MMP-7, MMP-11, MMP-1, MMP-8, MMP-3		Generation of a bioactive fragment from a1-proteinase inhibitor	Suppresses cancer-cell sensitivity to NK cells
MMP-7, MMP-8		Cleavage of the CC (β-chemokine) and CXC (α-chemokine) chemokine subfamilies	Regulate mobilization, leukocyte infiltration and migration

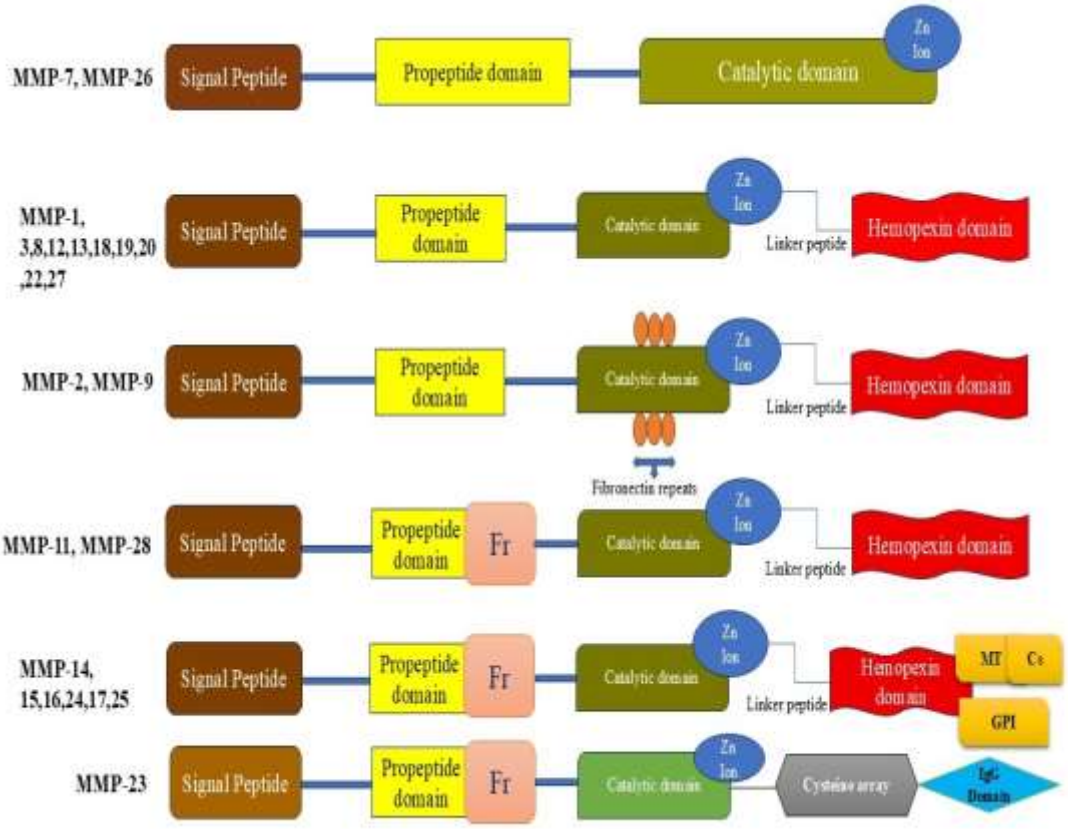


Fig. 1 Structure of MMPs

2.2 Importance of MMPs in Cancer Progression and Metastasis

Cancer is a group of diseases that are primary contributor of deaths globally. Many of the studies have shown that ECM remodeling proteases-Metrix metalloproteinases (MMPs) play a valuable role in the changes seen in the microenvironment during cancer advancement. (Page-McCaw et al., 2007). During the development of cancer, tumor cells communicate with the tumor microenvironment, including the growth factors, cytokines, and extracellular matrix and surrounding cells as macrophages, neutrophils and mast cells (Murphy, 2008), (Deryugina & Quigley, 2006). The four processes of cancer – migration, invasion, metastasis, and angiogenesis depend on this microenvironment. The MMPs expression in tumor microenvironment depends on cancer and stromal cells. MMPs have proteolytic activity and degenerate ECM physical barriers causing angiogenesis, invasion, and metastasis. The growth factors and cytokines signaling molecules cause tumor development. These factors are easily accessed by MMPs cancer microenvironment. This involves the cells acquiring

specific traits to escape the primary tumor, travel through the bloodstream, and form new tumors in distant organs. The process requires survival and communication skills from tumor cells. Overcoming physiological barriers is crucial for successful metastasis (Chambers et al., 2002, Pantel & Brakenhoff, 2004, Geho et al., 2005). At the stage of metastasis, tumor cells connect with various components like extracellular matrix, protein growth factors, and cytokines during metastasis. These interactions occur with different structures such as the basement membrane, blood vessels, and the microenvironment of secondary sites. These interactions contribute to the displacement of normal tissue and the metastatic foci formation.

MMPs have significant function in metastasis (Deryugina & Quigley, 2006,Quintero-Fabián et al., 2019). Regulation and dysregulation of MMPs in cancer involves various mechanisms that alter their expression, activation, and function. In many cancers, MMPs are often overexpressed, leading to increased degradation of ECM, which facilitates tumor invasion and metastasis. This upregulation can be mediated by various factors such as growth factors (e.g., TGF-β, EGF), cytokines (e.g., TNF-α), and oncogenic signaling pathways (e.g., MAPK, PI3K-Akt), cytokines and growth factors present in the microenvironment of tumor (Egeblad & Werb, 2002). DNA methylation and histone modifications can influence MMP expression patterns in cancer cells. For example, hypermethylation of promoter regions of certain MMP genes can lead to their silencing, while hypomethylation can contribute to their overexpression (Nagaset & Woessner, 1999). MMP activity can be modulated by post-translational modifications such as glycosylation, phosphorylation, and proteolytic processing. These modifications affect MMP activation, stability, and cell and ECM microenvironment localization. MicroRNAs (miRNAs) regulating MMP expression post-transcriptionally. Certain miRNAs can target MMP mRNAs for degradation or inhibit their translation, thereby modulating MMP levels in cancer cells, (Fabbri et al., 2007). TIMPs are endogenous inhibitors of MMPs that maintain the balance between MMP activity and ECM integrity. Dysregulation of TIMPs, either through reduced expression or increased degradation, can lead to excessive MMP activity and ECM degradation in cancer (Mustafa et al., 2022). The tumor microenvironment, characterized by hypoxia, inflammation, and interactions with stromal cells, influences MMP expression and activity. Hypoxia-inducible factors (HIFs) and

cytokines released by tumor-associated immune cells can upregulate MMP production, promoting tumor invasion and metastasis. (Sun, 2010).

2.3 Extracellular Matrix Remodeling by MMPs

The ECM is commonly composed of structural proteins (collagen and elastin), glycosaminoglycan, proteoglycan, and connecting proteins (fibronectin and laminin) (Yuan et al., 2023). The most common functions performed by the ECM are cell proliferation, differentiation, and maintenance of tissue homeostasis (Chakraborty & Edkins, 2021).

MMPs bind with the various ECM proteins involved in connective tissue remodeling (Laghezza et al., 2020). The remodeling of the ECM in many tumors has been connected with elevated expression of MMP-2, MMP-3, MMP-9, and MMP-14 (L. Luo et al., 2021). The degradation of collagen IV is responsible for the invasion of tumor cells into the basement membrane mediated by MMP-2 and MMP-9. It causes tumor metastasis and diffusion (Taleb et al., 2006). The collagen degradation also causes the remodeling of ECM biomechanical properties. The collagen dissolution around tumor cells is induced by MMP-14. It is a key contributor for cell invasion and migration (N. Chen et al., 2020).

2.4 MMP-Mediated Angiogenesis and Vasculogenesis

Angiogenesis and vasculogenesis are two common processes in cancer. Angiogenesis is the generation of new blood vessels from pre-existing ones (Bajbouj et al., 2021). Vasculogenesis is the process for the formation of new blood vessels through endothelial progenitor cells during embryonic development or in postnatal tissues under certain pathological conditions (Kovacic & Boehm, 2009). However, in cancer, it contributes to growth of tumor and metastasis by supplying vital substances and oxygen (Lugano et al., 2020).

MMPs are central to both angiogenesis and vasculogenesis, as they facilitate the remodeling of the ECM, which is important for the movement of endothelial cell, proliferation, and differentiation (Kubis & Levy, 2003). MMPs also modulate the

growth factor bioavailability and cytokines, thereby regulating the angiogenesis and Vasculogenesis (Mott & Werb, 2004).

MMP-2 and MMP-9 are important in angiogenesis and Vasculogenesis because of their ability to degrade type IV collagen, a basement membrane component. The basement membrane behaves as a barrier to cell migration, and its degradation by MMP-2 and MMP-9 is a key step for the new blood vessels formation (Shoari, 2024). The expression of these MMPs is frequently elevated when stimulated by pro-angiogenic signals, including VEGF and FGF, and transforming growth factor-beta (TGF-β) (Pathak et al., 2024). VEGF is a potent pro-angiogenic factor that stimulates EC proliferation, migration, and survival. MMP-9 has been shown to release VEGF from the ECM, increasing its bioavailability and enhancing its angiogenic effects. This interaction is crucial for the initiation of both angiogenesis and vasculogenesis, and for the formation of new vascular branches in the course of angiogenesis (Ghalehbandi et al., 2023).

TGF-β plays a bifunctional role in angiogenesis and vasculogenesis, pro-angiogenic and anti-angiogenic factor. TGF-β is secreted in a latent form bound to latency-associated peptide (LAP), which keeps it inactive. MMPs, particularly MMP-2 and MMP-9, can cleave LAP, releasing active TGF-β. The initiation of TGF-β by MMPs is for the regulation of angiogenesis by influencing EC proliferation and differentiation (Neel et al., 2012).

MMPs influence angiogenesis and vasculogenesis by modulating signaling pathways through the proteolytic processing of signaling molecules and receptors. Thus, MMPs can either activate or inactivate signaling pathways, for fine-tuning the angiogenic and vasculogenic response. It activates pro-MMP-2 by cleaving its Propeptide, converting it into the active enzyme that degrades type IV collagen and other ECM components (J. H. Chang et al., 2016).

MMPs can cleave VEGFR-2, modulating its activity and the downstream signaling pathways involved in EC proliferation and migration. This cleavage can result in either the activation of VEGFR-2 signaling or its inhibition, depending on the specific MMP involved. The regulation of VEGFR-2 by MMPs is critical for maintaining the

balance between angiogenesis and vasculogenesis, (X. Wang & Khalil, 2018), (Ceci et al., 2020).

2.5 MMP Inhibition Strategies

Strategies for MMP inhibition focus on designing and employing approaches that block or diminish MMP activity in order to manage or treat various diseases. MMP activity can be crucial in treating diseases where MMPs contribute to tissue damage, such as cancer, arthritis, and cardiovascular diseases.

2.5.1 Small Molecule Inhibitors of MMP Activity

Batimastat (BB-94) is a synthetic broad-spectrum small molecule that supresses the MMP activity including MMP-9. The Batimastat structure has a hydroxamate group that binds to the zinc ion at MMPs active site. This interaction is critical for the restriction of the enzyme proteolytic activity (Hernandez-Pando et al., 2000). Batimastat was administered in oral and intravenous routes. It interferes with ECM remodeling by attaching to the active site of MMPs, sequestering the zinc ion required for their function, and thereby blocking the breakdown of extracellular matrix components (Brew & Nagase, 2010).

Marimastat (BB-2516) is a next-generation oral broad-spectrum inhibitor, it inhibits MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 activity. The structure of Marimastat has a Hydroxymate, that function as a zinc chelator at the MMPs active site. Marimastat was studied in pancreatic, non-small cell lung, breast, colorectal, gastric, glioblastoma brain, and prostate cancer (Bramhall et al., 2002).

Other inhibitors including tanomastat Carboxylate zinc chelator, inhibits MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, prinomastat Hydroxymate zinc chelator inhibitor, inhibits MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14, and rebimastat (Winer et al., 2018), all these inhibitors were investigated in ovarian, pancreatic, lung, breast, and prostate carcinomas. These inhibitors demonstrated small inhibitory activity and failed in clinical trials for the positive effect on survival.

2.5.2 Antibody-Based Therapies Targeting MMPs

Antibody-based therapies targeting MMPs represent a promising approach to treating many diseases where MMP dysregulation is critical. Monoclonal antibodies (mAbs) are engineered proteins that can bind to specific antigens, such as MMPs, it is designed to selectively inhibit a single MMP with high affinity, greater specificity, reduced side effects (Alaseem et al., 2019).

The antib REGA-3G12 and REGA-2D9 are target MMPs (Liu & Khalil, 2017), (Fields, 2019). The REGA3G12 inhibits MMP-9 by affecting the catalytic domain and the N-terminal region, rather than the catalytic zinc ion of the fibronectin region (K. Li et al., 2020). Additionally, monoclonal antibodies AB0041 (Andecaliximab-GS-5745 humanized version with clinical trails) and AB0044 also target MMP-9 and have demonstrated the ability to inhibit tumor growth and metastasis through pro-MMP-9 activation and non-completely inhibits MMP-9 activity in colorectal carcinoma models.

2.6 Natural Compounds as MMP Inhibitors

Natural products are an important source of bioactive molecules for developing therapeutic applications. In some cases, it becomes approved as a drug (Newman & Cragg, 2012). Many numbers of the metabolites and small natural compounds are known for the inhibition of MMPs expression including MMP-2 and MMP-9 (Mudit & El Sayed, 2016), (Gentile & Liuzzi, 2017), (Eun Lee et al., 2019) including the flavonoids and polyphenols.

Kaempferol a polyphenol has anticancer, antidiabetic, anti-inflammatory, antiaging, and antiallergic properties (Imran et al., 2019). It prevents the nuclear translocation of the AP-1 transcription factor to the MMP-2 promoter, which suppresses the production of MMP-2 in human tongue carcinoma (SCC4 cells) and stops propagation and invasion (Lin et al., 2013). Thus, reducing cancer development and carcinogenesis (Lee et al., 2017). Naringenin has anti-inflammatory and anticancer activity extracted from fruits. It reduces the nuclear translocation of NF-κB transcription factor in MMP-2 and MMP-9 and controls inflammation and cancer metastasis (H. L. Chang et al.,

2017). Luteolin has been found to inhibit cell proliferation, metastasis, and angiogenesis and can sensitize cancer cells to therapeutic-induced cytotoxicity by suppressing phosphatidylinositol 3′-kinase (PI3K)/Akt and nuclear factor kappa B (NF-κB) and suppresses MMP-2 and MMP-9 expression in A375 human melanoma cells (Yao et al., 2019). Myricetin regulates MMP-2 and MMP-9 activity and reduces the MMP-2 production and expression in colorectal cancer cells (COLO 205). It reduces and inhibits metastasis in breast cancer cells (MDA-Mb-231) by reducing the expression of MMP-2 and MMP-9 activity (Ci et al., 2018). it also reduces the growth and propagation of lung cancer cells (A549-IR) by reducing MMP-2 and MMP-9 expression and stops the growth and movement of cancer (Kang et al., 2020).

Research conducted on quercetin flavonoids for its anti-inflammatory and anticancer activities which reduce propagation and invasion in human hepatocarcinoma cell lines (HCCLM3 cells). It suppresses MMP-2 and MMP-9 expression (Lu et al., 2018) in human oral cancer cells (HSC-6 and SCC-9) (Zhao et al., 2019). Genistein has antitumor, antibacterial, and antioxidant, properties. It inhibits angiogenesis and tumor cell programmed death. Silibinin stops skin cancer and affects metastasis in breast cancer by inhibiting the expression of MMP-9 in mice through suppression of the MEK/ERK cascade. It protects ECM by the control of MMP-9 expression in thyroid and breast cancer cell migration (Kim et al., 2009). Caffeic acid is an active transcription inhibitor and MMP-9 activity inhibitor were obtained from a plant *Euonymus alatus* (Kuo et al., 2015). Pterostilbene has antiproliferative, anti-inflammatory, anticancer, and antioxidant activities similar to Resveratrol, obtained from blueberries and other grape varieties (Rimando et al., 2002), (McCormack & McFadden, 2012).

2.7 Clinical Trials Assessing MMP Inhibition in Cancer Therapy

Matrix metalloproteinase inhibitors (MMPIs) ranged from normal, natural, and synthetic chelating agents. Many experiments and clinical trials support that MMPs participate in tumor invasion, angiogenesis, and metastasis, thus MMP acts as potential targets for cancer therapy. These experimental and therapeutic trials have been examined in several experimental models. The results of experiments and trials give the possibilities as classes of anticancer drugs.

Batimastat (BB-94) - Batimastat is a Hydroxymate (zinc chelator) type of inhibitor and explored MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-13) inhibitors as preclinical models (Chirvi et al., 1994). Batimastat inhibits the regrowth of human breast cancer (MDA-MB-435) in the mammary fat pads, metastasis of the lung (Sledge et al., 1995), growth of colon tumors, organ invasion, and metastasis. Batimastat has been tested on ovarian carcinomas, both alone and with traditional chemotherapy drugs (BROWN, 1994). Batimastat was the first explored MMP inhibitor, tested in an I-phase clinical trial and canceled in a Phase III clinical trial, due to low solubility and local toxicity. All the trials were stopped due to some general tissue reactions.

Marimastat (BB-2516) - Marimastat is a low-molecular-weight MMP including (MMP-1, MMP-2, MMP-7, and MMP-9) Peptidomimetic inhibitor that, has a similar action mechanism as Batimastat, with a 20% to 50 % oral bioavailability. The preclinical trial of Marimastat reached phases II and III in pancreatic, lung, breast, colorectal, brain, and prostate cancer (Levin et al., 2006), (Rosenbaum et al., 2005).

Prinomastat (AG 3340) - Prinomastat (AG 3340) is a Nonpeptidomimetic hydroxamic acid derivative MMP inhibitor that targets MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 and participates in tumor invasion and metastasis (Shalinsky et al., 1999). In advanced prostate cancer patients, the Phase I drug trial of Prinomastat (AG 3340) in association with mitoxantrone and prednisone is underway (Hidalgo & Eckhardt, 2001).

Rebimastat (BMS-275291) - Rebimastat (BMS-275291) is a broad-spectrum sulfhydryl-based mercaptoacyl (zinc chelator) targets MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP -14 in Phase I clinical research studies (Sikic, 1999). Rebimastat (BMS-275291) strong supress MMP-2 and MMP-9 activity.

Tetracycline Derivatives- The tetracycline derivatives can hamper the activity by binding with zinc and calcium ions of MMP (J. F. Fisher & Mobashery, 2006). The chemically modified tetracycline-like Doxycycline is the only FDA-approved MMP inhibitor that targets MMP-7 and MMP-8 (Kivelä-Rajamäki et al., 2003)

Doxycycline - Doxycycline act as an anticancer agent that can restrict the activity and production of several MMPs. It inhibits the secretion and activity of MMP-2 and

MMP-9 in MDA-MB-435 cancer cell lines culture. In in-vitro studies, it inhibits the growth and development of the U2OS osteosarcoma, PC-3 prostate, and MDA-MB-435 breast cancer cell lines. it also starts apoptosis and suppresses the invasion and metastatic of the MDA-MB-435 breast cancer and B16F10 melanoma cell lines, (Fife et al., 1998). In phase I medical studies on cancer patients, oral doses of 400 mg administered twice a day resulted consisted of fatigue, confusion, nausea, and vomiting as in dose-limiting toxicity (Nanda et al., 2016).

2.8 Natural MMP inhibition compounds

Neovastat (AE-941) - Neovastat (AE-941) orally administrated compound, has anti-angiogenic and anti-metastatic activity, and is isolated from shark cartilage. Many of the studies identified his effect on the inhibition of vascular endothelial growth factor (VEGF) and enzymatic activity of MMPs (FALARDEAU, 2001). The high-dose administration of neovastat in Phase I and Phase II clinical trials shows their survival benefit in cancer patients (F. E. Mott et al., 2003). The toxicity effects of neovastat are nausea, flatulence, diarrhea, vomiting, constipation, and rash.

Genistein is an isoflavonoid (polyphenol) that has anti-tumor, anti-inflammatory, and anticancer activity. It inhibits the activity of MMPs (MMP-2 and MMP-9) and tumor growth (X. Huang et al., 2005). In the case of breast and prostate cancers, there are several studies explaining that genistein has expressed a lower risk of cancer development and cancer patient death (Gu et al., 2005).

2.9 Conclusion

The creation of MMP inhibitors has number of challenges, like issues of selectivity, toxicity, lack of efficacy, pharmacokinetics, and biomarker identification. MMPs may have overlaying substrates and biological functions, thus inhibiting one MMP may not fully block the pathological process. This redundancy can reduce the potency of MMP inhibitors as therapeutic agents, in diseases like cancer, where multiple MMPs are involved in tumor progression and metastasis. Clinical trials of early MMP inhibitors, such as Marimastat, showed promising results. Advanced drug designing, targeted delivery systems, and biomarker discovery may eventually overcome these challenges and limitations, leading to more effective MMP-based therapies.

CHAPTER – 3

Synergistic Structural Inhibition of MMP-9 by Natural Flavonoids: A Natural Combinatorial Therapy against Cancer

3.1 Introduction

Cancers are a group of diseases connected with uncontrolled growth and rapid rise of unusual cells in the body. According to the W.H.O in 2022, there were an estimated 200 lakhs registered cancer and around 97 lakhs deaths due to cancer. Within in last five years, about a total of 53.5 million people received a cancer diagnosis. Globally, about 1 in 5 people develop cancer and approximately 1 in 9 men and 1 in 12 women die from the disease. Among cancers, the most widespread are lung cancer, breast cancer, and colorectum cancer. Lung cancer is a common death cause in men while in women, breast cancer is the most common cancer type (https://www. who.int/).

As per, National Cancer Registry Programme Report 2022, India, there were approximately 14,61,427 cancer cases, at the rate of 100.4 per 100,000. However, in children of age between 0 to 14 years, the most common cancer is lymphoid leukemia. In 2022, India recorded 1.4 million new cancer cases and 900,000 deaths due to cancers. Further, breast and cervix cancers were the most common female cancers accounting for 27% and 18% of new cases, respectively. Among males, lip and oral cavity cancers and lung cancers were the leading types, making up 15.6% and 8.5% of new cases, respectively (Sathishkumar et al., 2022). The WHO predicts a significant increase in new cancer cases, with a projected surge of 77% to over 35 million by 2050 (https://www.who.int/). This increase is attributed to lifestyle factors such as tobacco and alcohol use, obesity, population aging, and growth.

Matrix metalloproteinase-9 (MMP-9) is a Gelatinase B enzyme (Mondal et al., 2020). It is mainly secreted in the cerebellum, hippocampus, and cerebral cortex (Xiao et al., 2024a). The synthesis and secretion of MMP-9 occur in the form of inactive enzymes or as zymogens by endothelial cells, neutrophils, fibroblasts, and macrophages (Rashid & Bardaweel, 2023). At the time of granulocyte

differentiation, the bone marrow is the main site of its synthesis (Mondal et al., 2020). MMP-9 is connected with ECM degradation, tissue remodeling, and normal tissue turnover. The proteolytic activity of MMP-9 is in- volved in the alternation of cell-cell and cell-ECM interaction (Hsu et al., 2016). MMP-9 performs remodeling the basement membrane which is made by collagen type IV. Overexpression of MMP-9 has contributed to the progression of many diseases such as extracranial arteriovenous mal- formation (AVMs) (Rashid & Bardaweel, 2023), neurological diseases, and inflammatory processes [(Vafadari et al., 2016), (Hannocks et al., 2019), and cancers (H. Huang, 2018), (Li et al., 2017),(Akter et al., 2015). Enhanced expression of MMP-9 has been identified by various studies as a critical element in tumor development and progression (H. Huang, 2018), (Li et al., 2017),(Akter et al., 2015), (Amin et al., 2017). Tumor cells activate the neighboring cells that increase the production of MMPs by increasing the secretion of interferons, interleukins, and growth factors (Jabłońska-Trypuć et al., 2016). As a result, overexpression of MMP9 in cancer conditions results in basement mem- brane destruction (Hou et al., 2014), (Misko et al., 2002) and hence promotes tumor invasion, metastasis, angiogenesis, and intervening tumor microenvironment (Akter et al., 2015).

A past study showed that the MMP-9 polymorphism plays a essential role in breast cancer and also helps in the identification of individuals with high risk (Rashid & Bardaweel, 2023). In colon cancer, overexpression of avb6 integrin was found, that increases MMP-9 secretion, followed by protein-kinase pathway activation (Niu et al., 1998). VEGF stimulates the expression of MMP-9 in lung cancer, which contributes to enhanced metastatic potential (Hiratsuka et al., 2002). The suppression of MMP-9 by matrix metalloproteinase inhibitors (MMPIs) at the catalytic site can ameliorate the dreaded consequences of MMP9 over- expression (Amin et al., 2017). Inhibition of MMP9 suppresses cell proliferation by inducing apoptosis through the release of ligands, such as TNFa and TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand), from their membrane-bound inactive form (Nyormoi et al., 2003).

Therefore, overexpression of MMP-9 has a strong association with a wide range of cancers and their progression, so MMP-9 can be considered as a potential target to

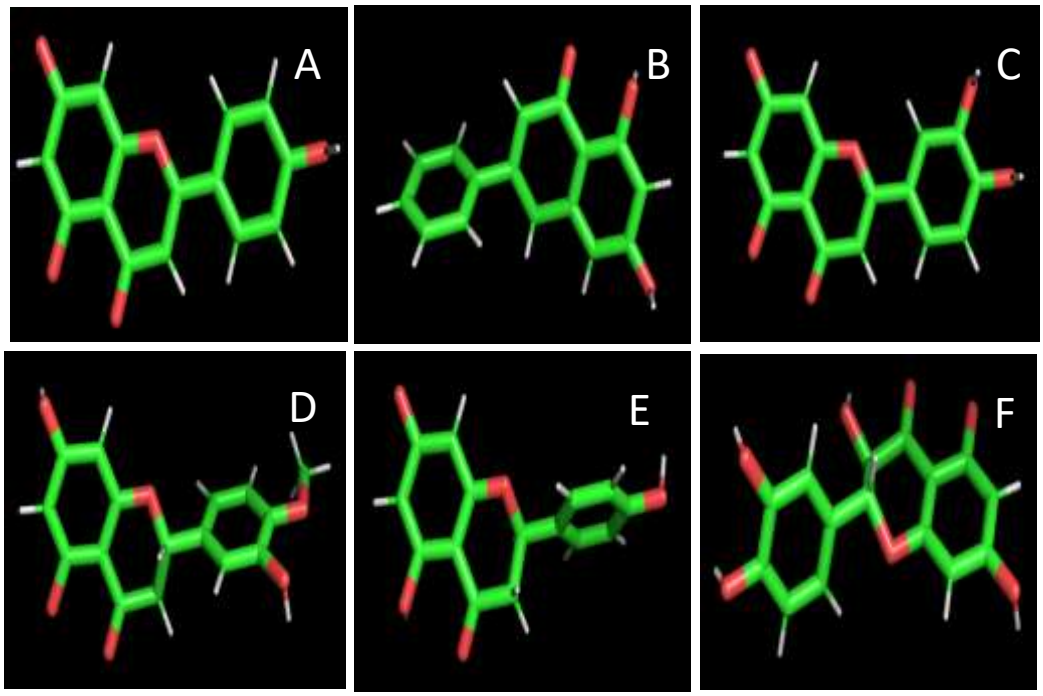
develop effective therapies against cancer.

Flavonoids, is a polyphenolic compound widely distributed in fruits, vegetables, and herbs, have antioxidant and anti-inflammatory properties, their potential to support health, and their relatively low toxicity. Several studies have been done to evaluate the structural inhibition potential of flavonoids against different targets of cancers.

Rathod *et al.* in their *in-silico* investigation of natural anti-cancer agents, identified Gancaonin Q as a potential anticancer agent (Rathod et al., 2023). Rajiv Gandhi *et al.* identified chlorogenic acid as an effective inhibitor of MMP-9 (Rajiv Gandhi et al., 2024). Kumari & Kumar, in their study on glioblastomas identified 7,4′-dihydroxyflavan, 4′-hydroxy-7-methoxyflavan, and (3R)-3-(4-hydroxybenzyl)-6-hydroxy-8-methoxy-3,4-di- hydro-2H-1-benzopyran flavonoids as significant inhibitors of MMP-9 (Kumari & Kumar, 2023). Synergistic effects of drug combinations have been observed to display more effective inhibition in several infectious diseases against the target proteins but few studies on the utilization of a synergistic combination of flavonoids have been conducted against cancer (De Forni et al., 2022), (Gupta et al., 2022), (Wiraswati et al., 2024), (Ajji et al., 2020)

3.2 Methodology

3.2.1 ADMET Analysis



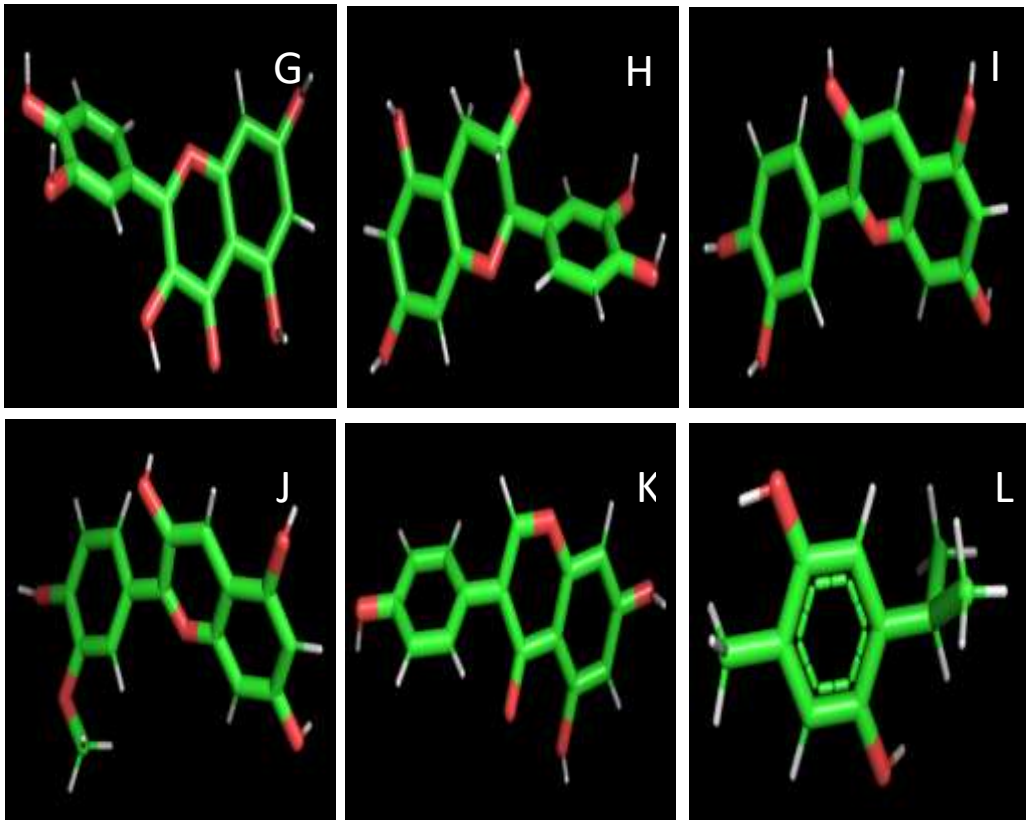
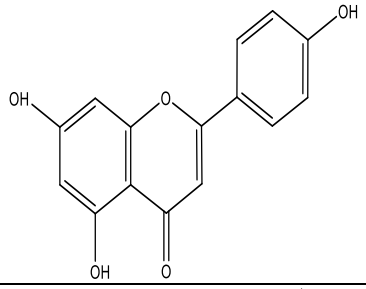
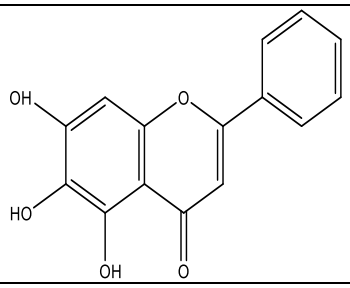
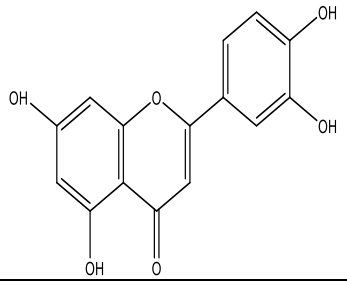
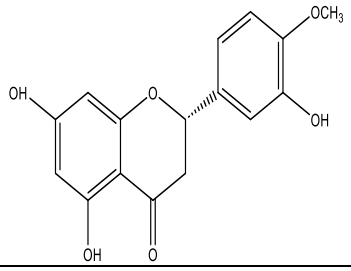
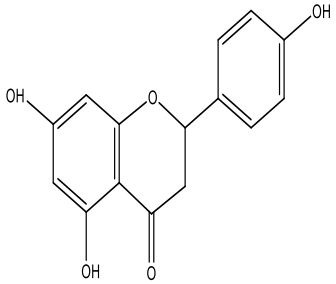


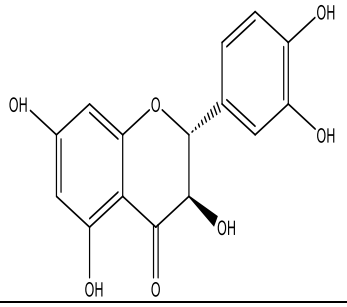
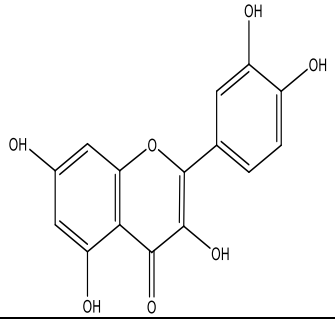
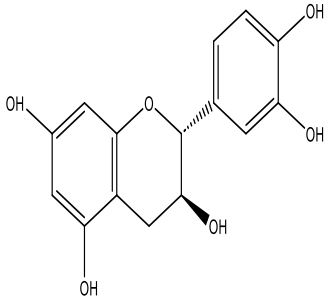
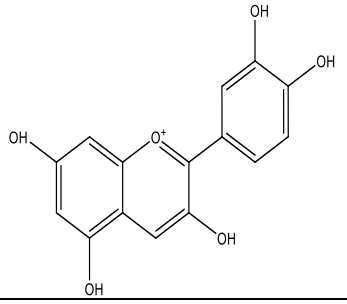
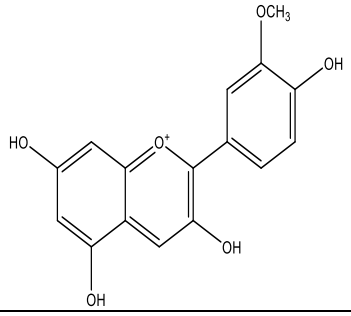
Fig. 2: - 3D View Flavonoids: (A) Apigenin, (B) Chrysin, (C) Luteolin, (D) Hesperetin, (E) Naringenin, (F) Taxifolin, (G) Quercetin, (H) Catechin, (I) Cyanidin, (J) Peonidin, (K) Genistein, (L) Thymohydroquinone

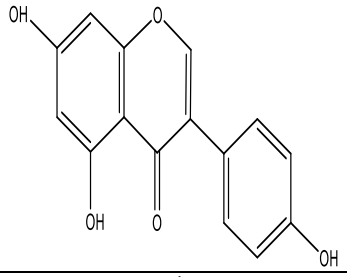
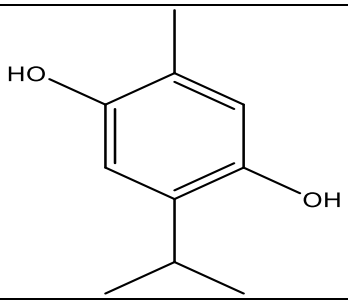
Flavonoids like Apigenin, Chrysin, Luteolin, Hesperetin, Naringenin, Taxifolin, Quercetin, Catechin, Cyanidin, Peonidin, Genistein, and Thymohydroquinone (Table 2) were selected based on previous literature and their low toxicity profiles for investigating their potential against cancer. The structures of the selected Flavonoids were drawn, using ChemDraw Ultra Version 12.0 for the stereochemistry, and converted into SMILES format. To evaluate the physiochemical properties, toxicity, and bioactivity of the considered compounds Swiss ADME (Daina et al., 2017), ProTox II (Banerjee et al., 2018), and Molinspiration (https://www.molinspiration.com/) web servers were used respectively. Further, to validate the ADME properties ADMElab3.0 (https://admetlab3.scbdd.- com/server/evaluationCal) server was used.

The comparative analysis is completed by a chord diagram for their different properties using the Origin 2023b academic version (https://www.originlab.com/).

Table 2: – Chemical structure and PubChem CID of selected Flavonoids.

S. No.	Compound Name	PubChem CID	Chemical Structure
1	Apigenin	5280443	
2	Chrysin	5281607	
3	Luteolin	5280445	
4	Hesperetin	72281	
5	Naringenin	439246	

6	Taxifolin	439533	
7	Quercetin	5280343	
8	Catechin	73160	
9	Cyanidin	128861	
10	Peonidin	441773	

11	Genistein	5280961	
12	Thymohydroquinone	95779	

3.2.2 Principal Component Analysis (PCA)

The Principal Component Analysis (PCA) is used for the analysis of the correlation between the physiochemical, bioactivity, and toxicity properties (including Heavy Atom, Atom Number, Aromatic Atoms, Mol. Wt., Log P, H-Donor, H-Acceptor, Rotatable Bonds, Molar Refractivity, TPSA, Formal charge, Rings, Toxicity class, GPCR ligand, Ion channel modulator, Kinase Inhibitor, Nuclear Receptor Ligand, Protease Inhibitor, Enzyme Inhibitor) of the considered flavonoids by using the Minitab trial version 2021.

3.2.3 Molecular Docking

The 3D structure (Fig. 3) coordinate file of the MMP-9 (PDB ID-6ESM) was procured from the Protein Data Bank (RCSB). The 3 dimensional structures of all considered flavonoids were generated through an online smile translator tool (https://cactus.n- ci.nih.gov/translate/). To explore the synergistic effect of flavonoids both single ligands and combinations of ligands were docked at the active site of MMP-9. For combination docking, both ligands in each combination were docked simultaneously by preparing different ligand combinations in respective files (Raghavendra et al., 2015). The addition of Kollman charges to the protein structure were -20.664 atomic units. The used grid size for the docking had grid center: X = -2.6087, Y = 49.4802, Z = 17.5705; grid size: X = 15.545, Y= 16.283, Z= 22.504, and grid spacing of 0.375. Docking was performed using

the Lamarckian genetic algorithm, with a population size set to 150, the number of evaluations as 25,00000, and the number of generations were 27,000. The rate of crossover and gene mutation were 0.8 and 0.02 respectively. The binding energies of individual ligand docking were compared with the docking of combinations of ligands and the best combinations which showed favorably high negative binding energy compared to their counterparts were considered the most effective synergistic flavonoid combinations that can effectively mediate structural inhibition of MMP-9. To further validate the binding energies and interactions, all the compounds were docked 100 times each individually. The combination dockings of all flavonoids were then performed with the number of iterations as 10 and then the obtained two best combinations of flavonoids were docked 100 times independently in a site-specific manner. Average values and standard deviations of binding energies of 100 independent dockings of all ligands individually and the two best combinations were analyzed and the most consistent interactions with high negative binding energies were considered.

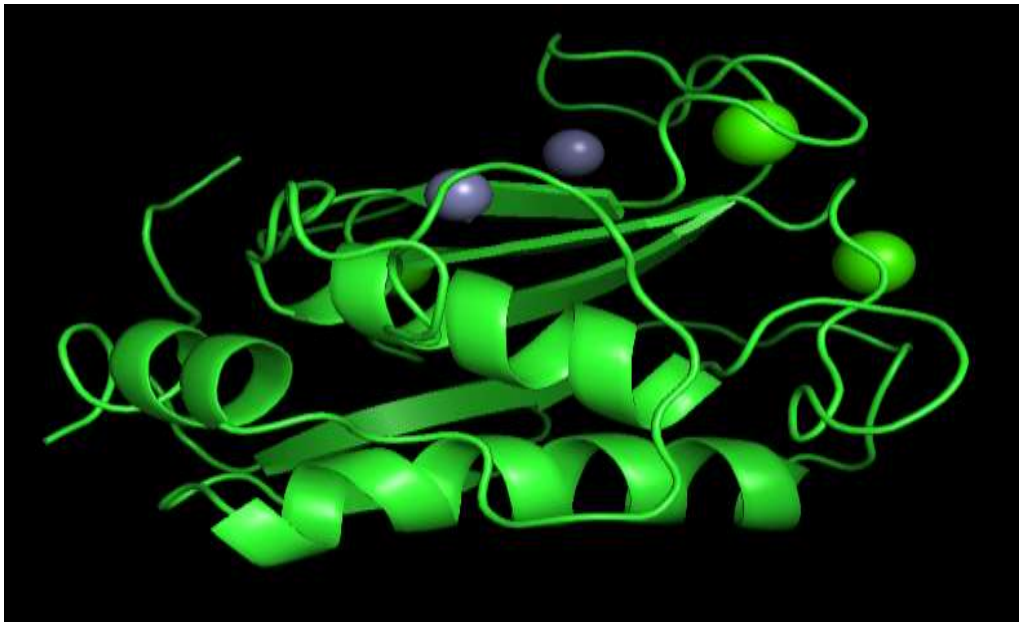


Fig. 3: Molecular View of MMP-9 (PDBID-6ESM)

3.3 Results

The present study deals with the analysis of natural flavonoids that can potentially inhibit MMP-9, a lead target for treating the adverse pathologies of cancer. The present study initially analyzed the physiochemical, bioactivity, and toxicity

properties of the flavonoids. Along with this their MMP-9 inhibition potential both individually and in combi- nations was studied using a molecular docking approach. All 12 selected flavonoids including Apigenin, Chrysin, Luteolin, Hesperetin, Naringenin, Taxifolin, Quercetin, Catechin, Cyanidin, Peonidin, Genistein, and Thymohydroquinone were docked individually and in combinations of two against MMP-9 (PDB ID-6ESM) to find the synergistic inhibition of the considered target of cancer.

3.3.1 ADMET Analysis

The ADMET examination of the considered 12 flavonoids (Apigenin, Chrysin, Luteolin, Hesperetin, Naringenin, Taxifolin, Quercetin, Catechin, Cyanidin, Peonidin, Genistein, and Thymohydroquinone) by using different tools gave consistent results and determined the effective physiochemical properties, toxicity values, and bioactive properties of the flavonoids. All the compounds followed Lipinski's rule of 5 (Lipinski et al., 2001) with no violation thus displaying high drug-likeness of the flavonoids in concern. The LD_{50} values of all the compounds ranged in toxicity class 3 to 5 with most of the compounds lying in class 5 thus proving the least toxicity (Table 3 & 4, Fig. 4). The bioactivity scores showed negative scores for the maximum number of the flavonoids for GPCR ligands, ion channel modulators, protease inhibitors, and kinase inhibitors proving the least off target inhibition by these com- pounds.

3.3.2 PCA Analysis

Principal component analysis was performed to identify the correlation between the physiochemical, bioactivity, and toxicity properties of the flavonoids. The first two components defined the maximum variance (61.2% for the first component and 14.5% for the second component) among the observations for the considered variables or properties. Cumulatively the first two components define 75.6% of the variance (Fig. 5). The scree plot displays the eigenvalues of the correlation of different components and thus displays the amount of explained variance defined by each component (Fig. 5). The score plot indicates that the catechins and taxifolin have similar properties compared to others. Luteolin has similar physiochemical and bioactive properties to that of quercetin (Fig. 5).

Table 3: - ADMET properties of selected Flavonoids were analysed by using Swiss ADMET.

S. No.	Molecule	Heavy Atom	Atom Number	Aromatic Atoms	Mol. Wt.	Log P	H-Donor	H-Acceptor	Rotatable Bonds	Molar Refractivity	TPSA	Formal Charge	Rings	Lipinski Rule violation	Ghose vi-olation	Veber violation	Rule of 3 violation	Reos violation	LD_{50} (mg/Kg)
1	Apigenin	20	20	16	270.053	2.577	3	5	1	72.914	90.9	0	3	0	0	0	1	0	2500
2	Chrysin	19	19	16	254.058	2.871	2	4	1	71.25	70.67	0	3	0	0	0	1	0	3919
3	Luteolin	21	21	16	286.048	2.282	4	6	1	74.579	111.13	0	3	0	0	0	1	0	3919
4	Hesperetin	22	22	12	302.079	2.519	3	6	2	76.747	96.22	0	3	0	0	0	2	0	2000
5	Naringenin	20	20	12	272.068	2.51	3	5	1	70.195	86.99	0	3	0	0	0	1	0	2000
6	Taxifolin	22	22	12	304.058	1.186	5	7	1	73.249	127.45	0	3	0	0	0	2	1	2000
7	Quercetin	22	22	16	302.043	1.988	5	7	1	76.244	131.36	0	3	0	0	0	2	1	159
8	Catechin	21	21	12	290.079	1.546	5	6	1	72.623	110.38	0	3	0	0	0	1	1	10000
9	Cyanidin	21	21	16	287.055	2.909	5	5	1	74.381	112.45	1	3	0	0	0	1	1	5000
10	Peonidin	22	22	16	301.071	3.212	4	5	2	79.268	101.45	1	3	0	0	0	2	0	5000
11	Genistein	20	20	16	270.053	2.577	3	5	1	72.914	90.6	0	3	0	0	0	1	0	2500
12	Thymohydroquinone	12	12	6	166.099	2.53	2	2	1	48.598	40.46	0	1	0	0	0	0	2	1000

Table 4: - Bioactivity scores of the selected Flavonoids estimated by the Molinspiration online server.

S. No.	Molecule	GPCR ligand	Ion channel modulator	Kinase Inhibitor	Nuclear Receptor Ligand	Protease Inhibitor	Enzyme Inhibitor
1	Apigenin	-0.07	-0.09	0.18	0.34	-0.25	0.26
2	Chrysin	-0.12	-0.18	0.19	0.17	-0.35	0.26
3	Luteolin	-0.02	-0.07	0.26	0.39	-0.22	0.28
4	Hesperetin	0.04	-0.26	-0.20	0.38	-0.13	0.16
5	Naringenin	0.03	-0.20	-0.26	0.42	-0.12	0.21
6	Taxifolin	0.09	0.03	-0.04	0.29	0.05	0.29
7	Quercetin	-0.06	-0.19	0.28	0.36	-0.25	0.28
8	Catechin	0.41	0.14	0.09	0.60	0.26	0.47
9	Cyanidin	-0.13	-0.09	0.02	0.09	-0.30	0.01
10	Peonidin	-0.16	-0.18	0.01	0.03	-0.34	-0.04
11	Genistein	-0.22	-0.54	-0.06	0.23	-0.68	0.13
12	Thymohydroquinone	-0.92	-0.44	-1.06	-0.54	-1.17	-0.46

The biplot contains the information from both the loading plot and the score plot. It shows the bioactivity properties of the components. while most of the physiochemical properties mostly have positive coefficients for the first component but less positive or negative coefficients for the second component (Fig. 5).

3.3.3 Molecular Docking

The docking studies of individual flavonoids with MMP-9 exhibited that luteolin has the highest negative binding energy (-10.04 kcal/mol) followed by apigenin (-9.674 kcal/mol) with MMP-9, (Table 5). The formation of hydrogen bond defines effective interactions indicated that the majority of the flavonoids formed hydrogen bonds with MMP-9 at the active site residues. Results indicated that luteolin, quercetin, and catechin established four hydrogen bonds, apigenin, naringenin, taxifolin, cyanidin, and genistein formed three hydrogen bonds, hesperetin, and peonidin resulted in two hydrogen bonds with the active site of the target protein. (Table 5 and Figs. 7). The residues of AAs that established hydrogen bonds with the selected ligands are

GLN227, ALA189, LEU188, PRO240, ALA242, VAL223, MET247, ARG249, PRO246, but TYR245 amino acid residue formed a hydrogen bond with thymohydroquinone and genistein. The binding energies of all the flavonoids ranged from -10.04 kcal/mol for luteolin to -7.166 for naringenin and the inhibition constants (*Ki*) ranged from 0.0437 to 5.5884µM (Table 5). Thus, most of these flavonoids possess the potential to inhibit MMP-9, which has an important role in cancers.

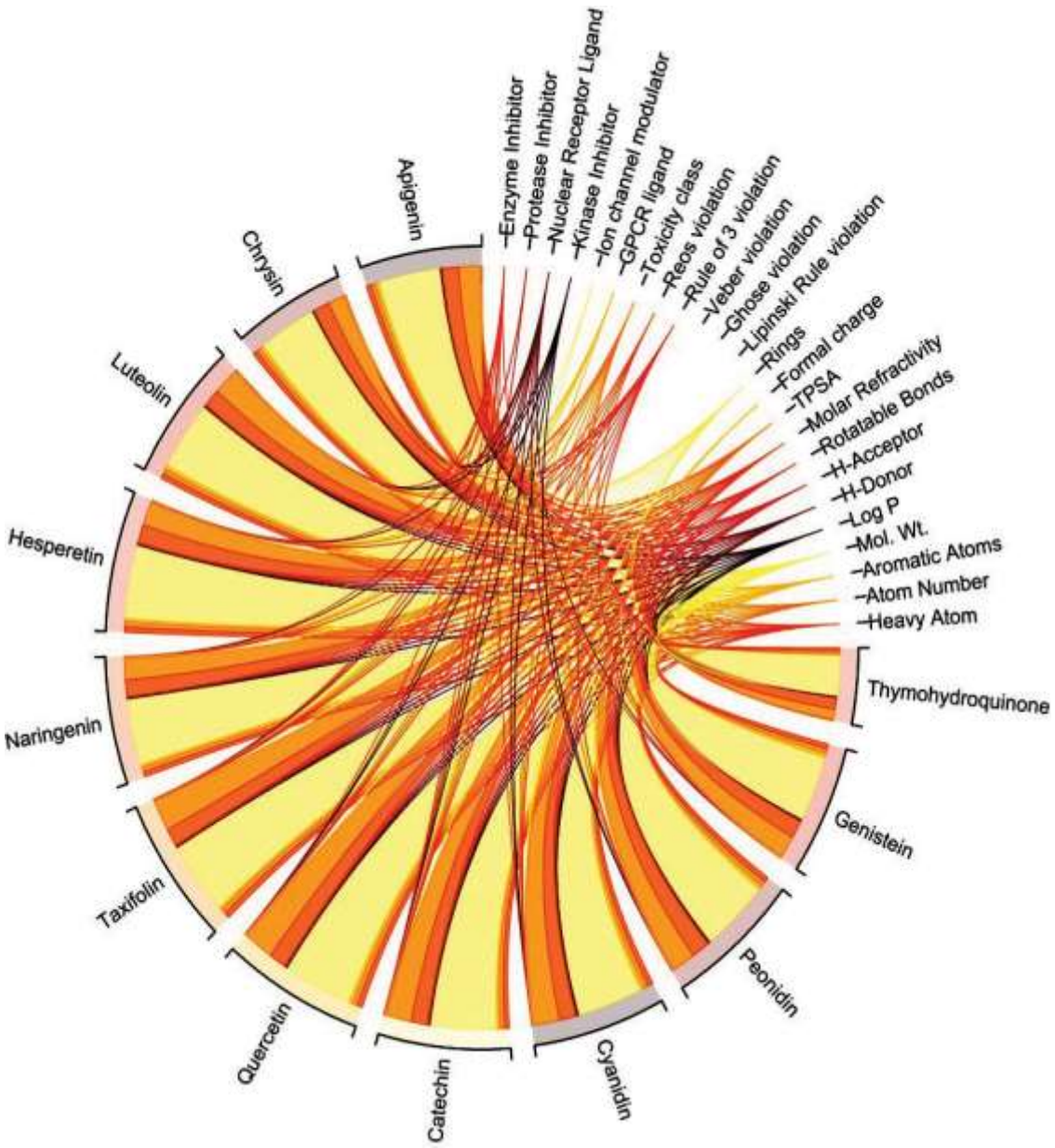
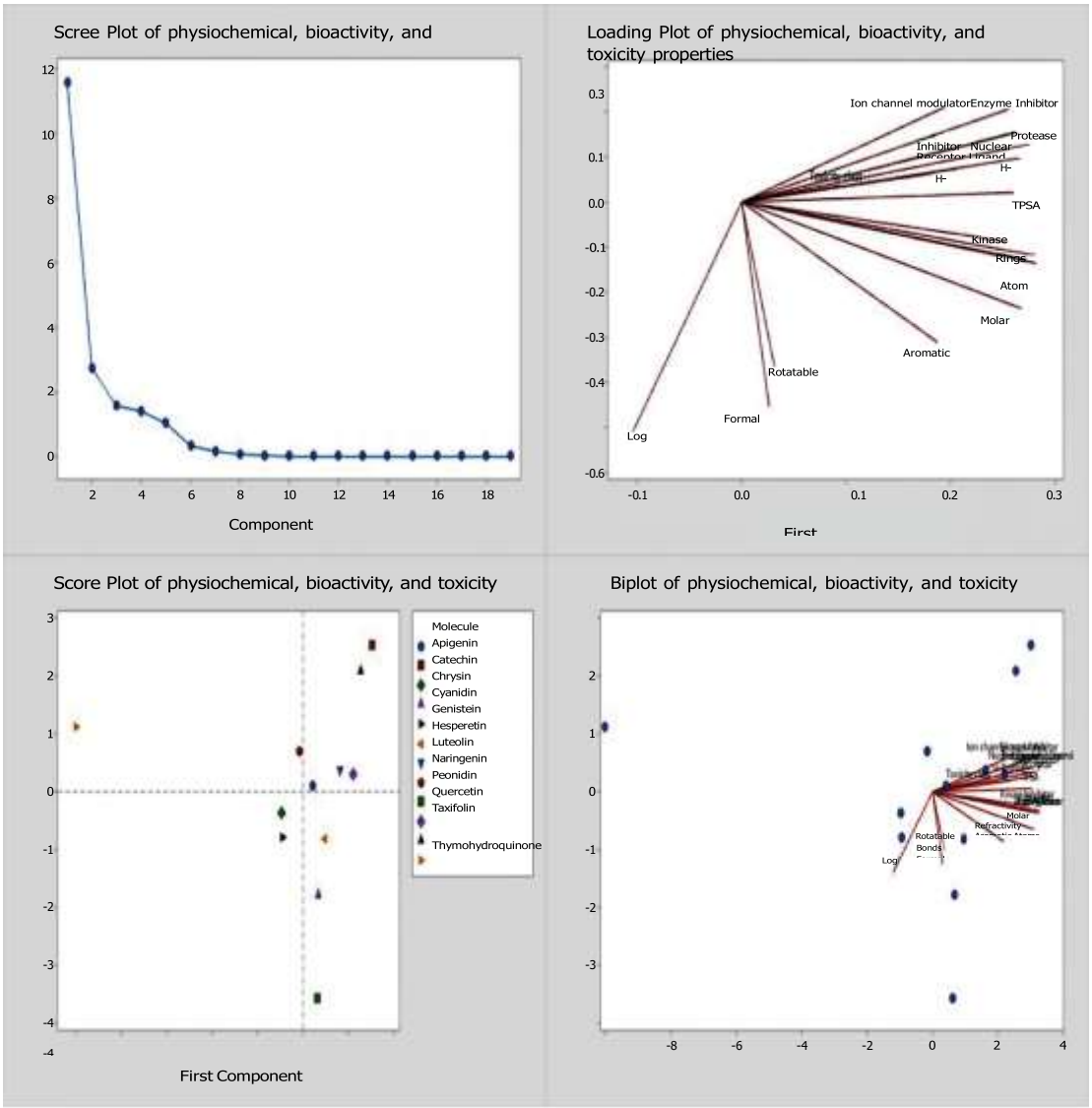


Fig. 4: - Chord diagram showing distribution of different physiochemical, bioactivity and toxicity properties of selected 12 flavonoids.



Eigen analysis of the Correlation Matrix

Eigenvalue	11.619	2.748	1.574	1.408	1.036	0.338	0.163	0.074	0.036	0.003	0.000
Proportion	0.612	0.145	0.083	0.074	0.055	0.018	0.009	0.004	0.002	0.000	0.000
Cumulative	0.612	0.756	0.839	0.913	0.968	0.985	0.994	0.998	1.000	1.000	1.000
Eigenvalue	0.000	0.000	0.000	0.000	0.000	0.000	-0.000	-0.000			
Proportion	0.000	0.000	0.000	0.000	0.000	0.000	-0.000	-0.000			
Cumulative	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			

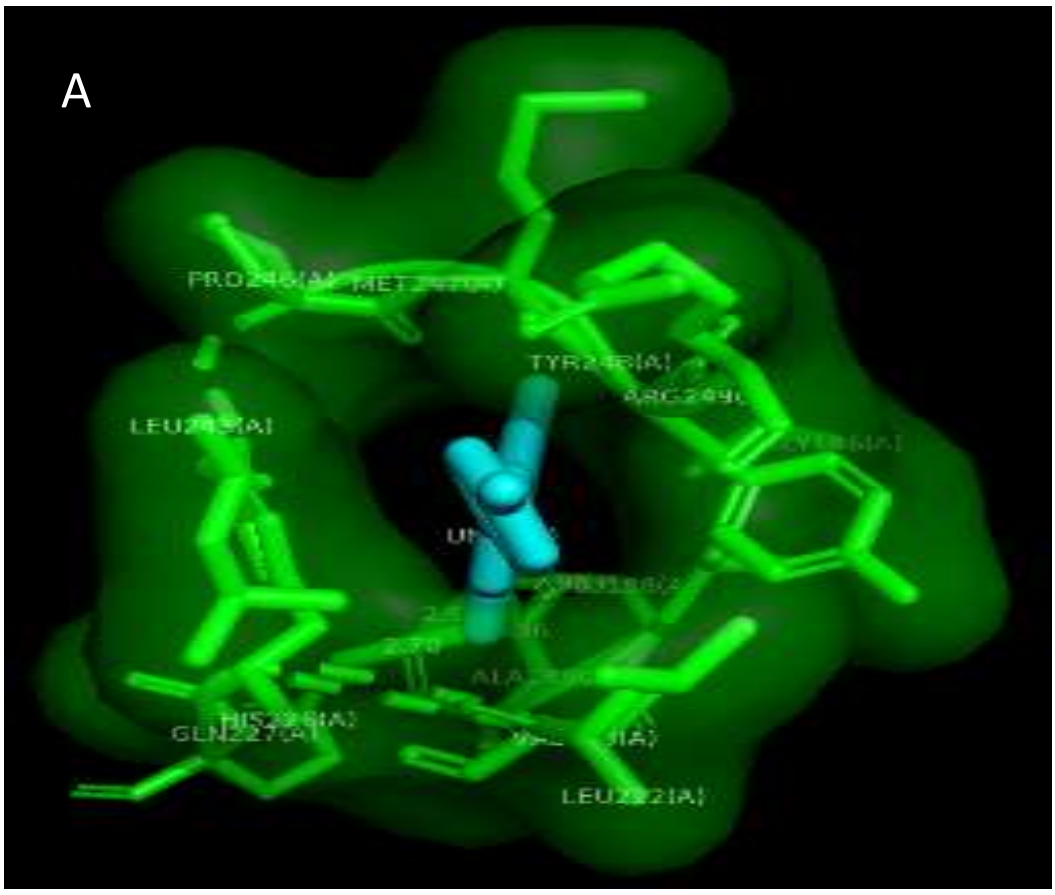
Fig. 5: - PCA analysis of physiochemical, bioactivity and toxicity properties of the 12 considered flavonoids showing A) Scree plots, B) Score plot, C) Loading plot and D) Biplot.

Table. 5: - Amino acids interaction, Hydrogen bond formation, and Binding energies of MMP-9 (6ESM) – with selected Flavonoids.

S. No.	Ligands with 6ESM	Amino acids interaction	Hydrogen bond formation with Amino acid	Hydrogen bond length	Binding energies (kcal/mol)	Ki (µM)
1	Apigenin	LEU243(A), ARG249(A), HIS226(A), LEU222(A), **GLN227(A,** VAL223(A), **ALA189(A),** GLY186(A), **LEU188(A),** PRO246(A), TYR248(A), MET247(A)	**GLN227(A) ALA189(A), LEU188(A)**	2.70 2.96, 2.90 2.98	-9.674	0.0811
2	Chrysin	VAL223(A), TYR248(A), LEU222(A), LEU243(A), THR251(A), ALA242(A), GLU241(A), **PRO240(A),** ARG249(A), TYR245(A), MET247(A), HIS226(A), GLN227(A), PRO246(A)	**PRO240(A)**	2.71	-9.45	0.1183
3	Luteolin	**LEU188(A),** VAL223(A), **ALA189(A), GLN227(A,** LEU222(A), ARG249(A), **ALA242(A),** MET247(A), LEU243(A), HIS226(A), TYR248(A), TYR245(A), PRO246(A)	**LEU188(A), ALA189(A), GLN227(A) ALA242(A)**	3.11 2.95, 2.96 2.79 3.11	-10.04	0.0437
4	Hesperetin	GLY186(A), **ALA189(A),** LEU188(A), VAL223(A), LEU222(A), **GLN227(A,** ARG249(A), LEU243(A), MET247(A), TYR248(A), TYR245(A),	**ALA189(A), GLN227(A)**	2.90, 2.97 2.71	-8.23	0.9276

		HIS226(A), PRO246(A),				
5	Naringenin	ARG249(A), MET247(A), LEU222(A), **GLN227(A,** VAL223(A), **ALA189(A),** LEU187(A), **LEU188(A),** GLY186(A), PRO246(A), HIS226(A), TYR248(A), TYR245(A), LEU243(A)	**GLN227(A) ALA189(A), LEU188(A)**	2.75 2.88, 3.10 2.80	-7.166	5.5884
6	Taxifolin	LEU243(A), MET247(A), TYR248(A), LEU222(A), **GLN227(A,** VAL223(A), **LEU188(A),** LEU187(A), **ALA189(A),** GLY186(A), PRO246(A), TYR245(A), HIS226(A)	**GLN227(A) , LEU188(A), ALA189(A)**	3.21, 2.62 2.69 3.02	-7.412	3.6895
7	Quercetin	**TYR245(A),** LEU243(A), LEU222(A), HIS226(A), PRO246(A), GLY186(A), LEU187(A), **ALA189(A), LEU188(A), GLN227(A,** MET247(A), TYR248(A), VAL223(A),	**ALA189(A), ALA189(A), LEU188(A), GLN227(A)**	3.12 2.93 2.69 3.21, 2.65	-7.965	1.4508
8	Catechin	LEU243(A), LEU222(A), TYR245(A), **VAL223(A),** HIS226(A), GLN227(A), **ALA189(A), LEU188(A),** LEU187(A), PRO246(A), GLY186(A), TYR248(A), **MET247(A,** ARG249(A)	**VAL223(A), ALA189(A), LEU188(A), MET247(A)**	3.19 2.87, 3.11 2.80 3.13	-7.348	4.1103

9	Cyanidin	GLN227(A), VAL223(A), LEU188(A), TYR248(A), LEU222(A), GLU241(A), LEU243(A), THR251(A), TYR245(A), MET247(A), ALA242(A), **PRO240(A), ARG249(A, PRO246(A),** HIS226(A)	**PRO240(A), ARG249(A) PRO246(A)**	2.90 3.06 2.70	-9.601	0.0917
10	Peonidin	**ARG249(A,** TYR245(A), **PRO240(A),** GLU241(A), PRO246(A), HIS226(A), HIS236(A), MET247(A), GLN227(A), VAL223(A), LEU222(A), TYR248(A), THR251(A), LEU243(A), ALA242(A)	**ARG249(A) PRO240(A)**	3.22 2.82	-9.11	0.2101
11	Genistein	GLN227(A), **ALA189(A),** VAL223(A), TYR248(A), HIS226(A), LEU222(A), THR251(A), ARG249(A), LEU243(A), ALA242(A), **TYR245(A),** MET247(A), **LEU188(A)**	**ALA189(A), TYR245(A), LEU188(A)**	3.33 2.80 3.20	-9.54	0.1017
12	Thymohydroquinone	LEU222(A), TYR248(A), ARG249(A) ALA242(A) LEU243(A) **TYR245(A)** MET244(A) MET247(A) HIS226(A) VAL223(A) PRO246(A) LEU188(A) GLN227(A)	**TYR245(A)**	3.06	-7.5	8.2113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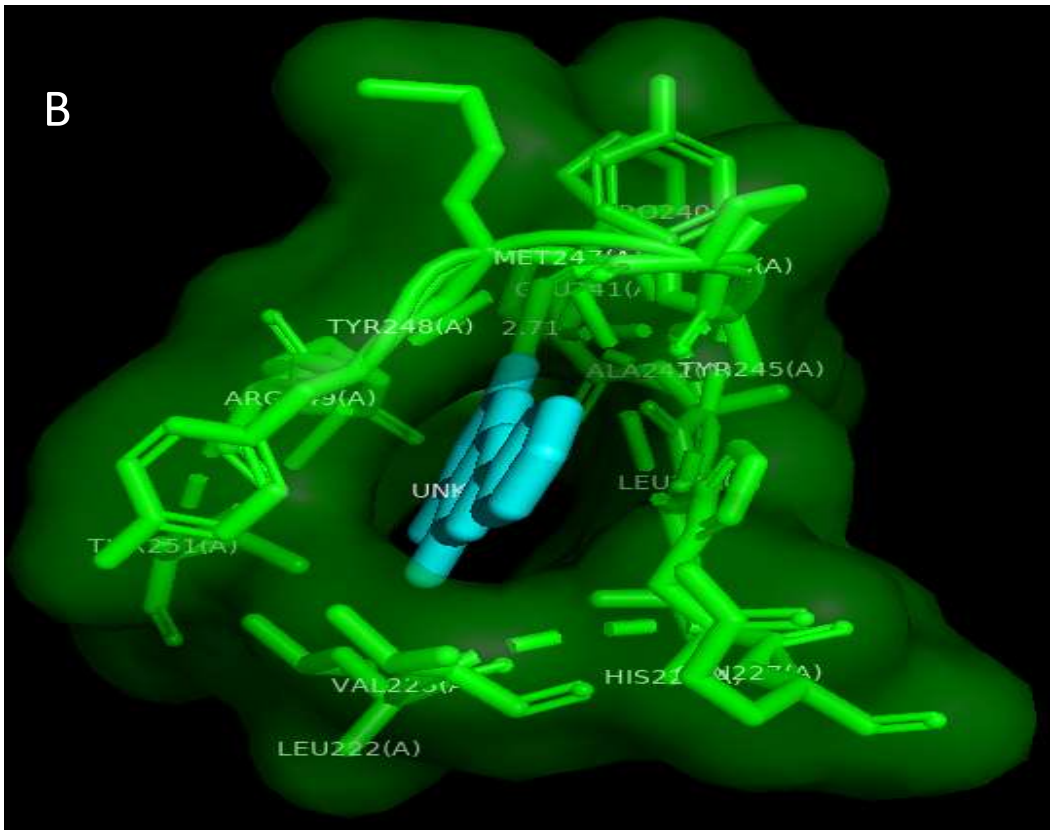
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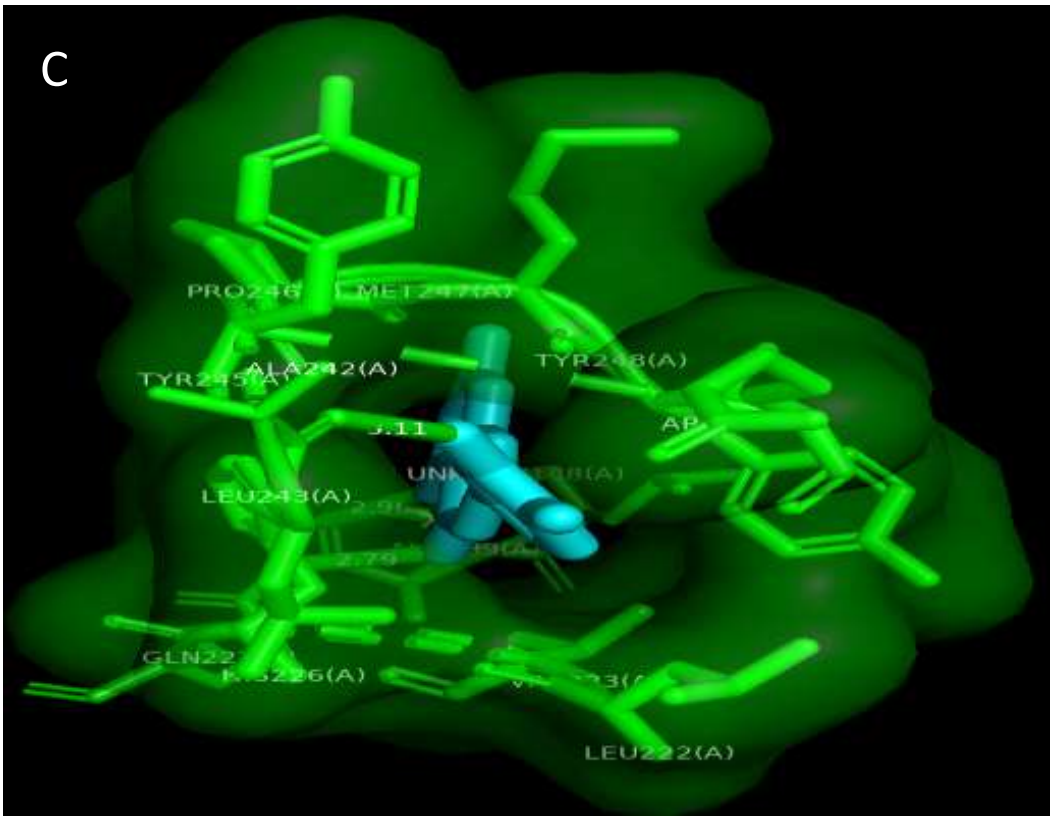
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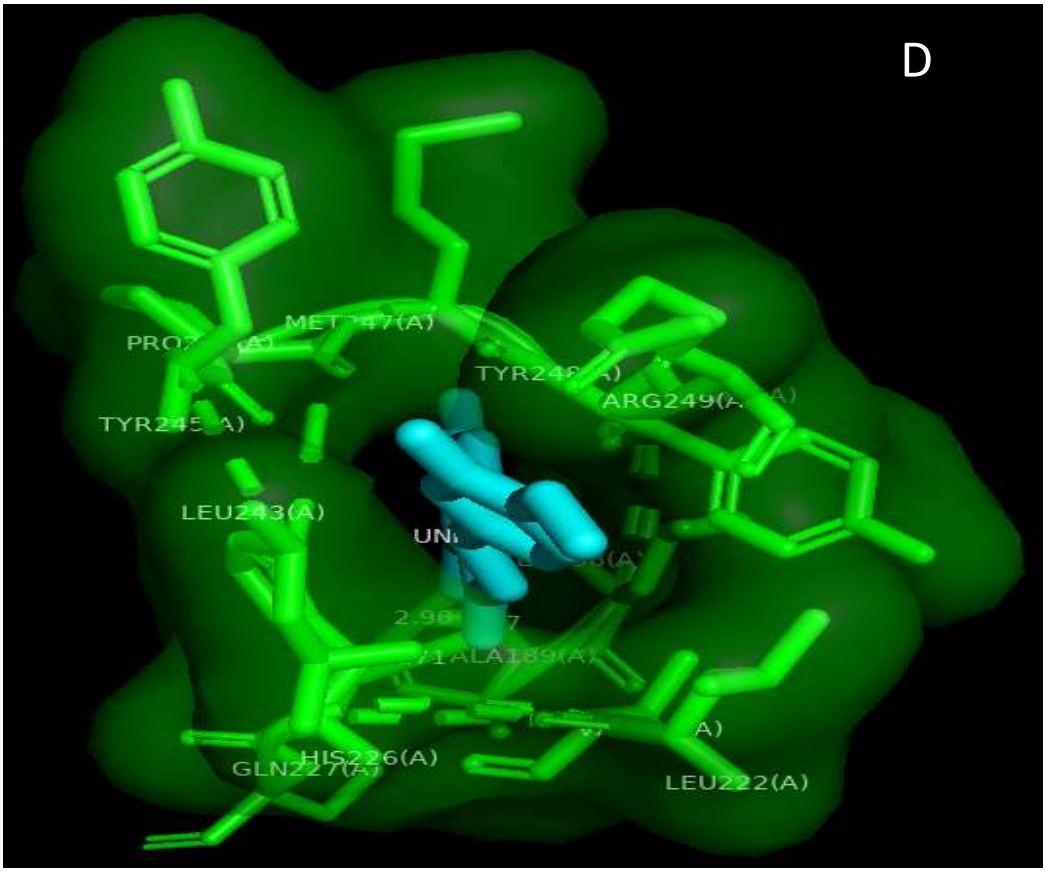
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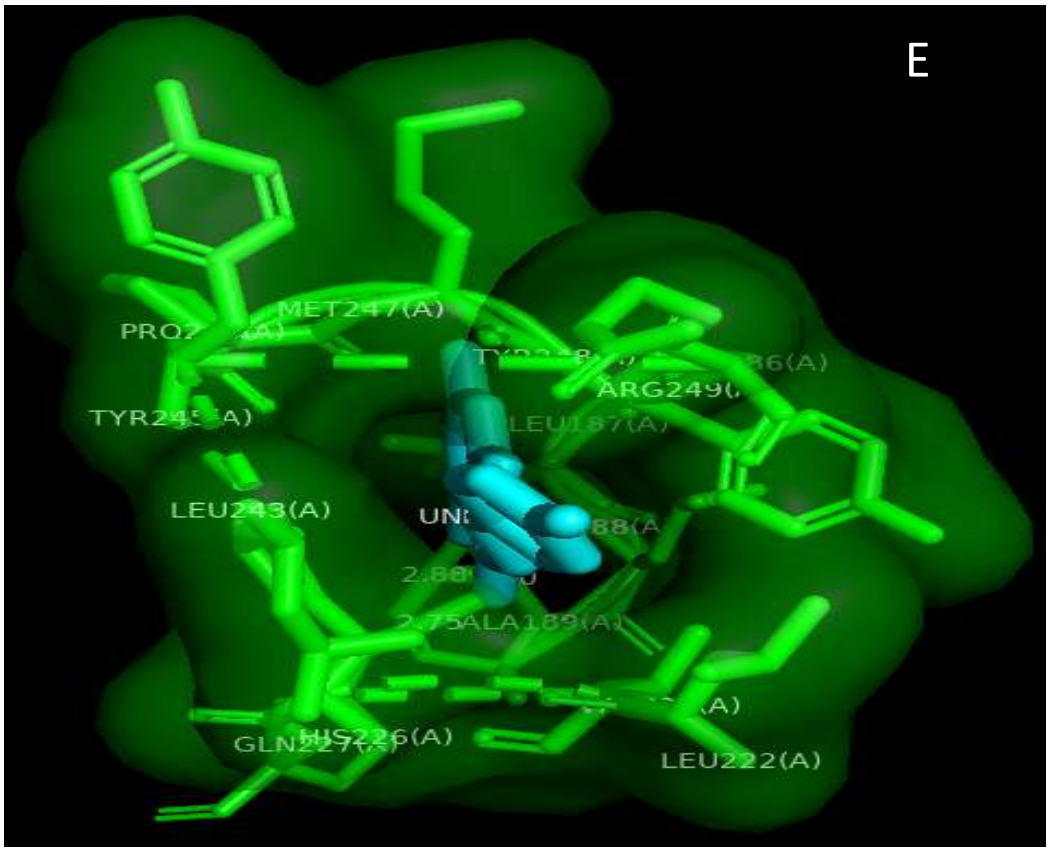
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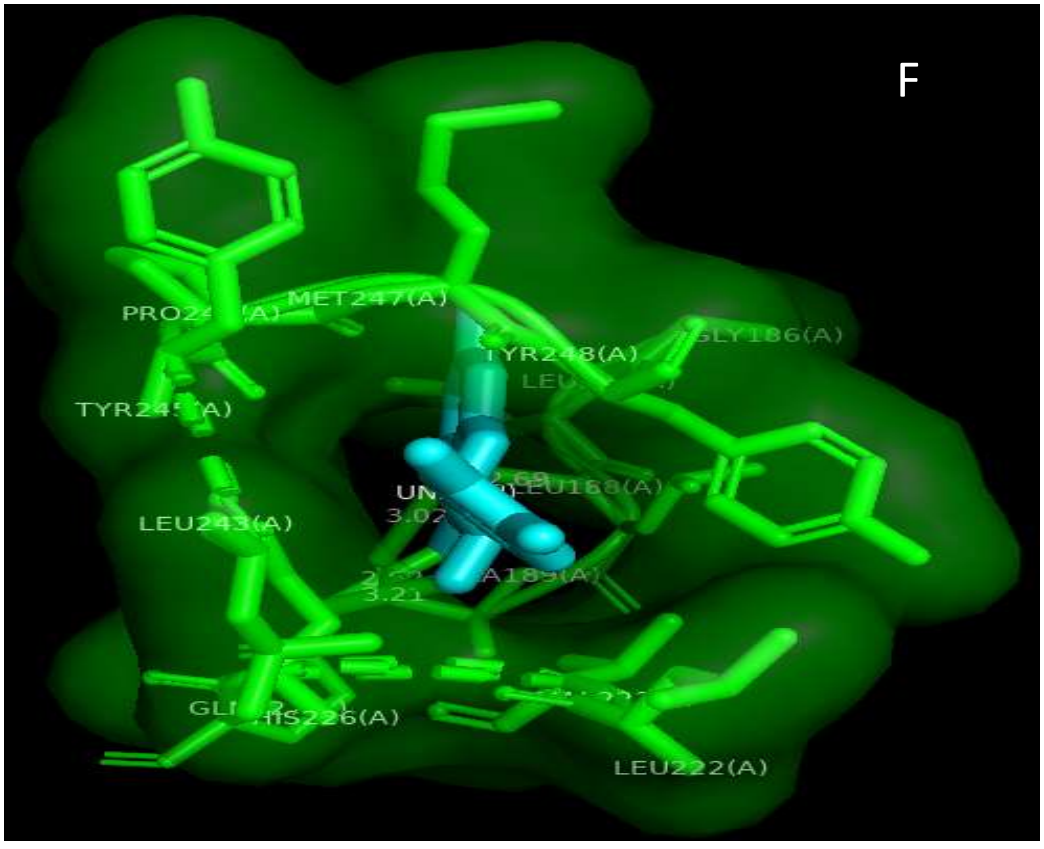
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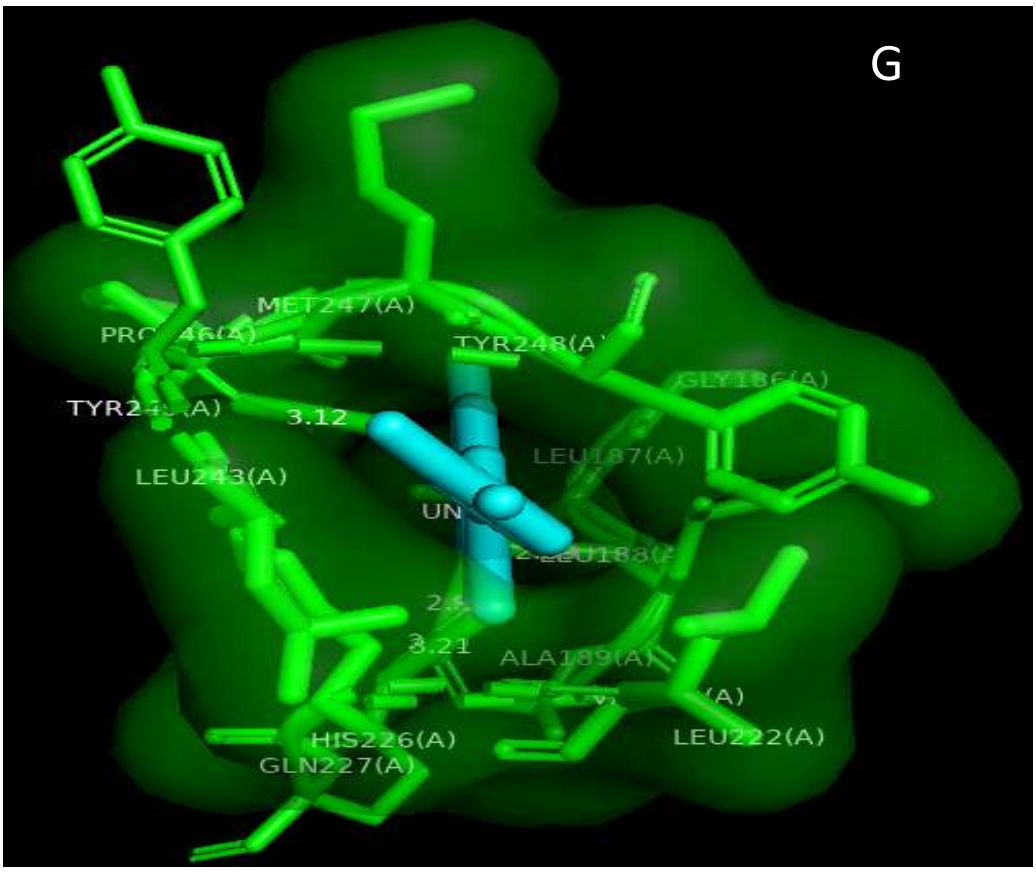
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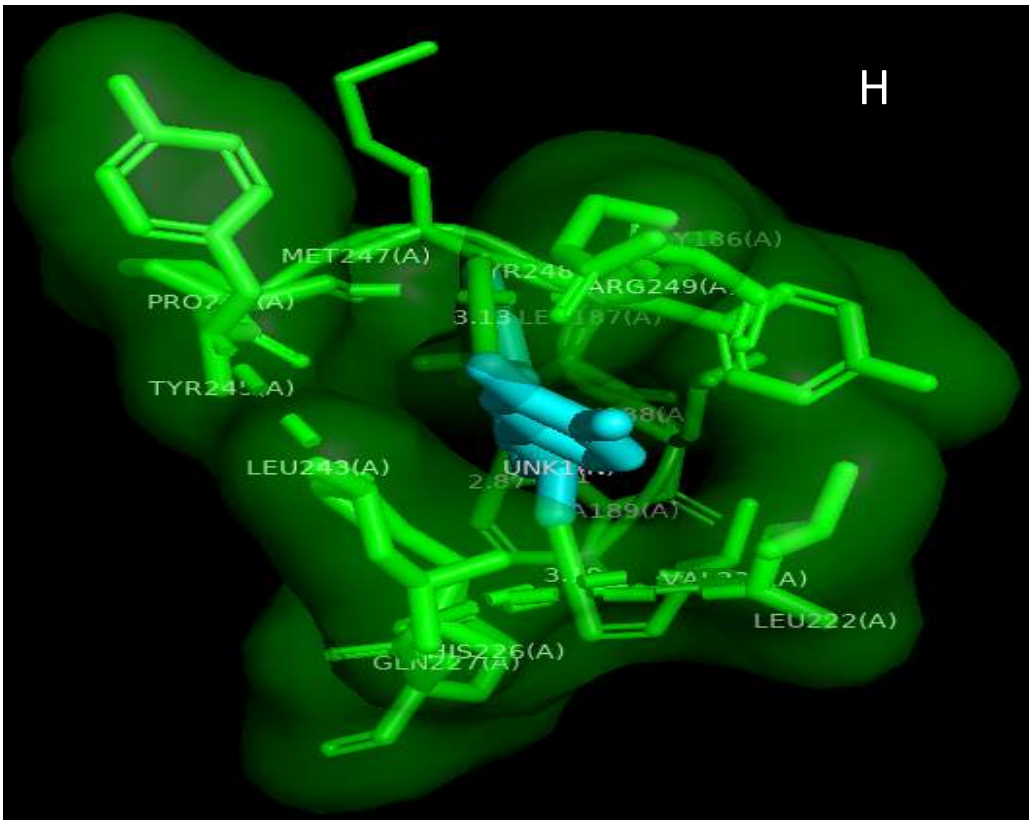
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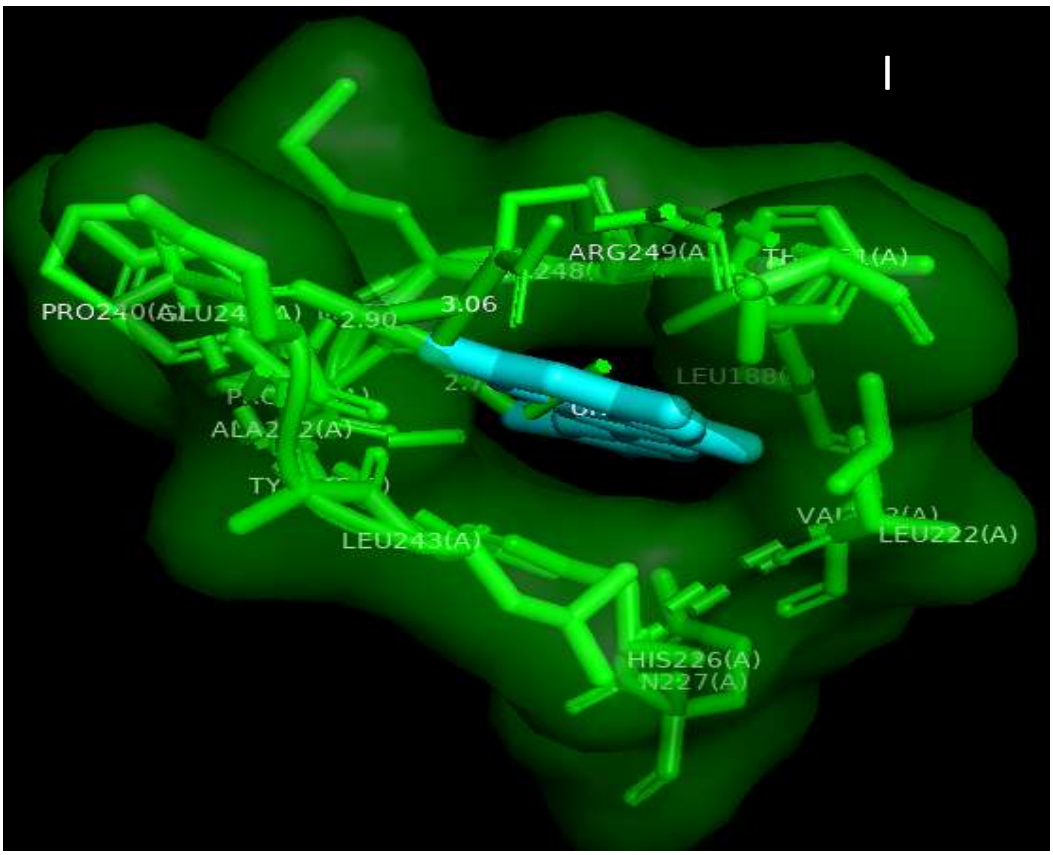
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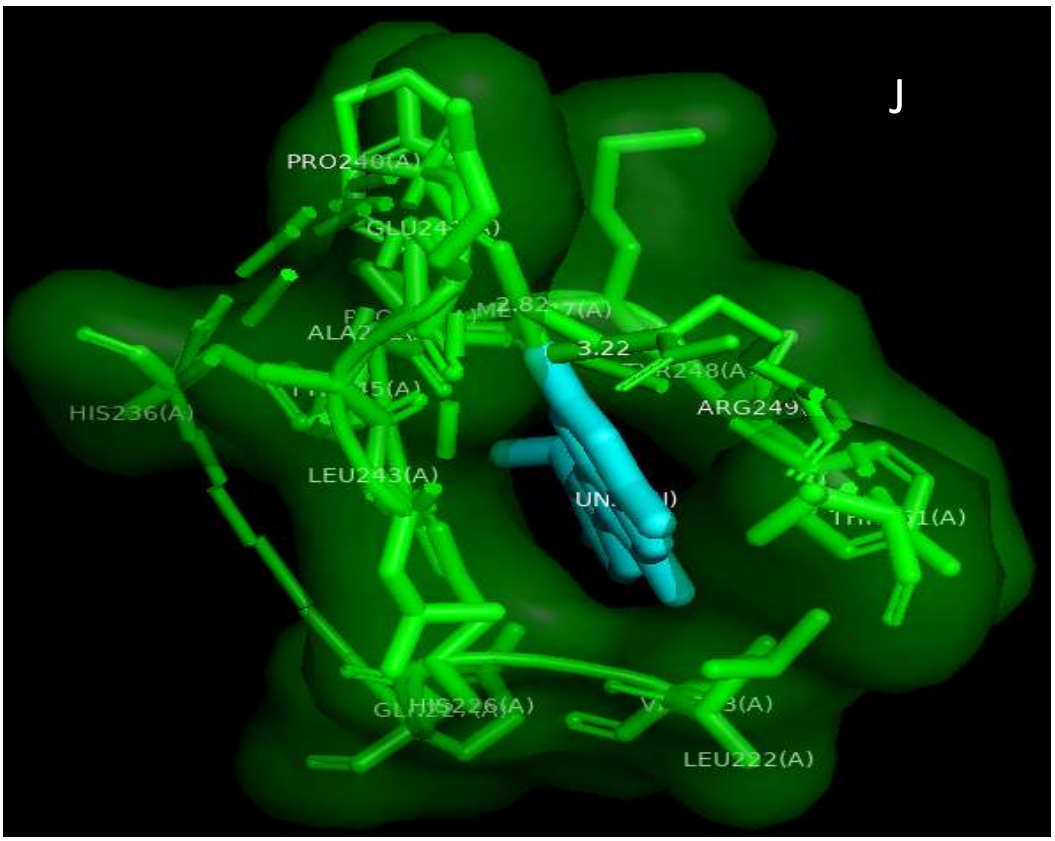
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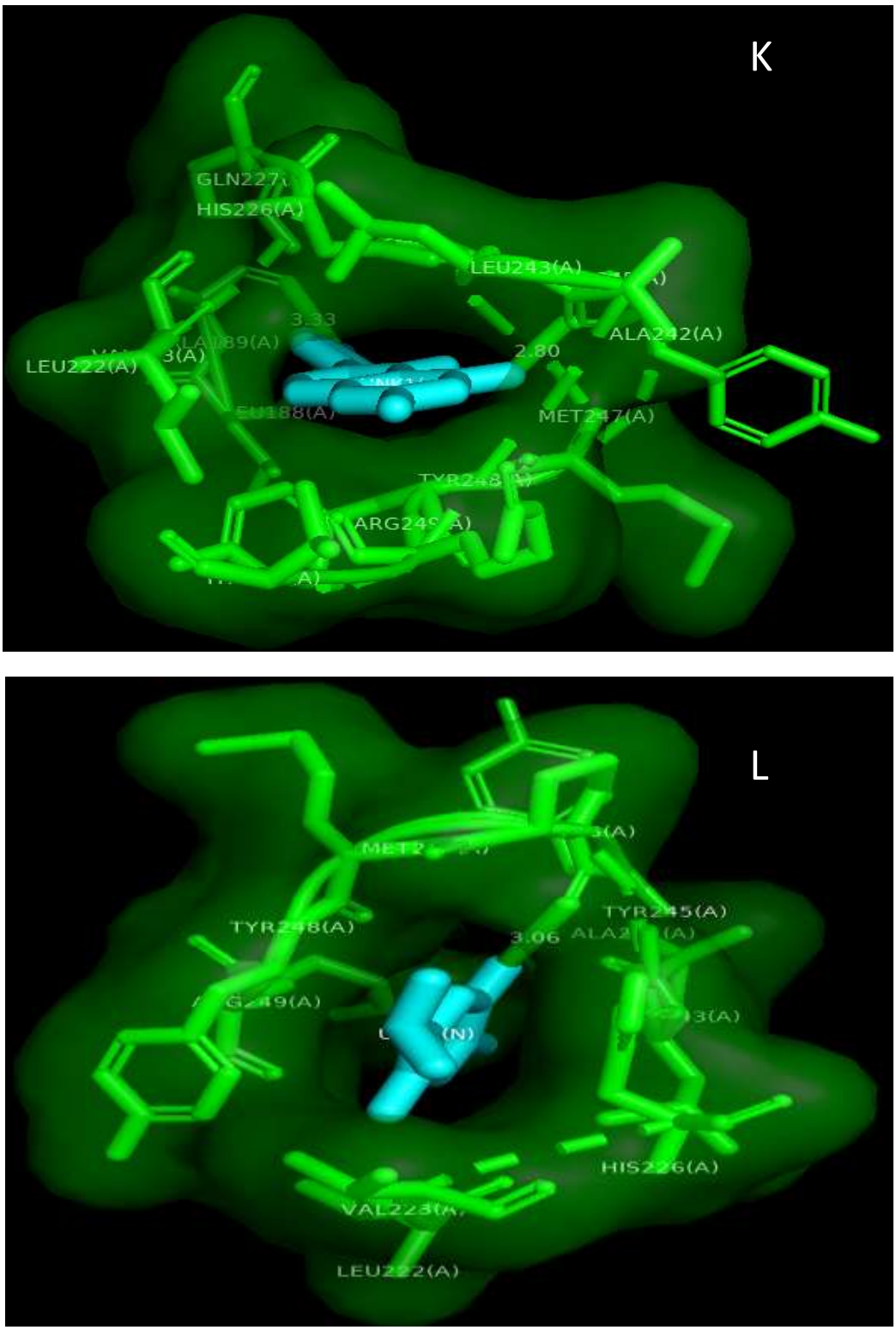
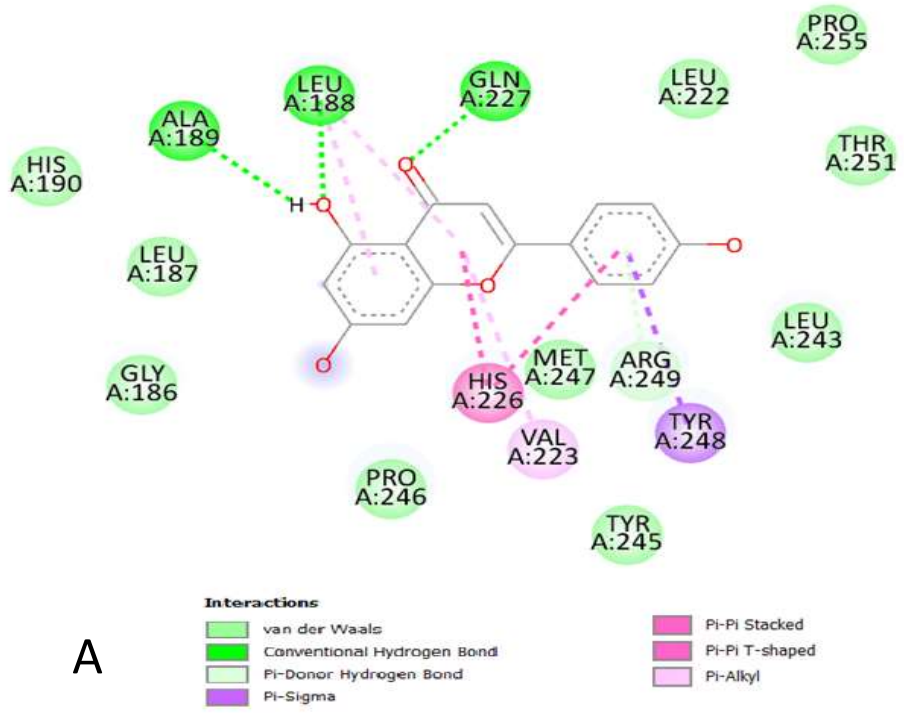


Fig.6: - 3D Molecular docking conformations of MMP-9 (6ESM) with Flavonoids: (A) Apigenin, (B) Chrysin, (C) Luteolin, (D) Hesperetin, (E) Naringenin, (F) Taxifolin, (G) Quercetin, (H) Catechin, (I) Cyanidin, (J) Peonidin, (K) Genistein and (L) Thymohydroquinone.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Pi-Donor Hydrogen Bond
Pi-Sigma
Pi-Pi Stacked
Pi-Pi T-shaped
Pi-Alkyl

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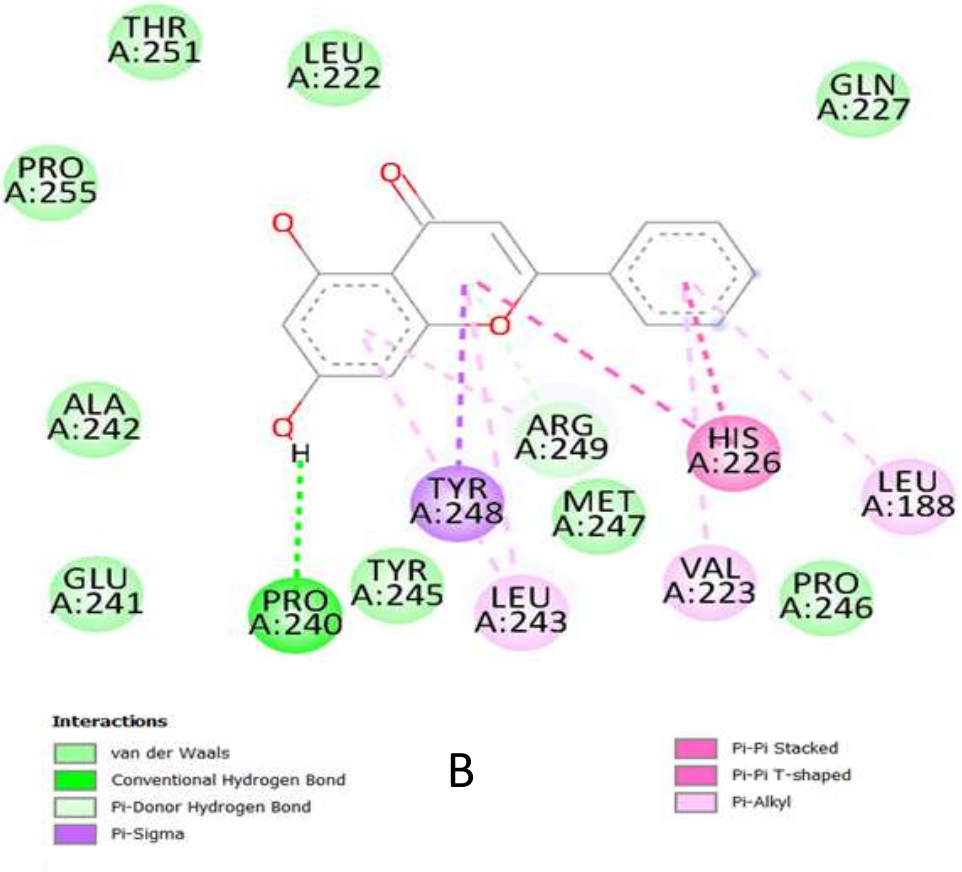
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Pi-Pi T-shaped
Pi-Alkyl

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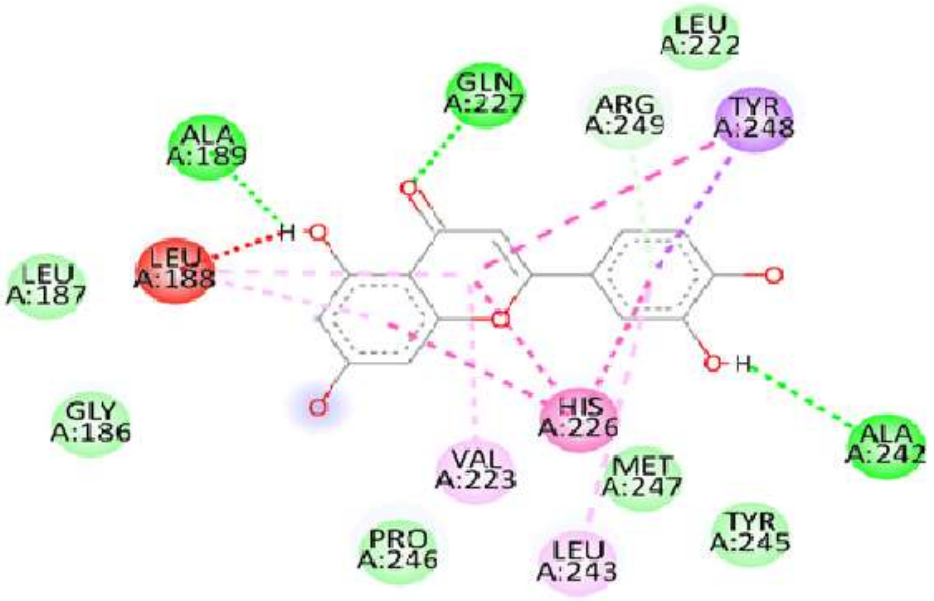
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Interactions
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Pi-Pi T-shaped
Pi-Alkyl

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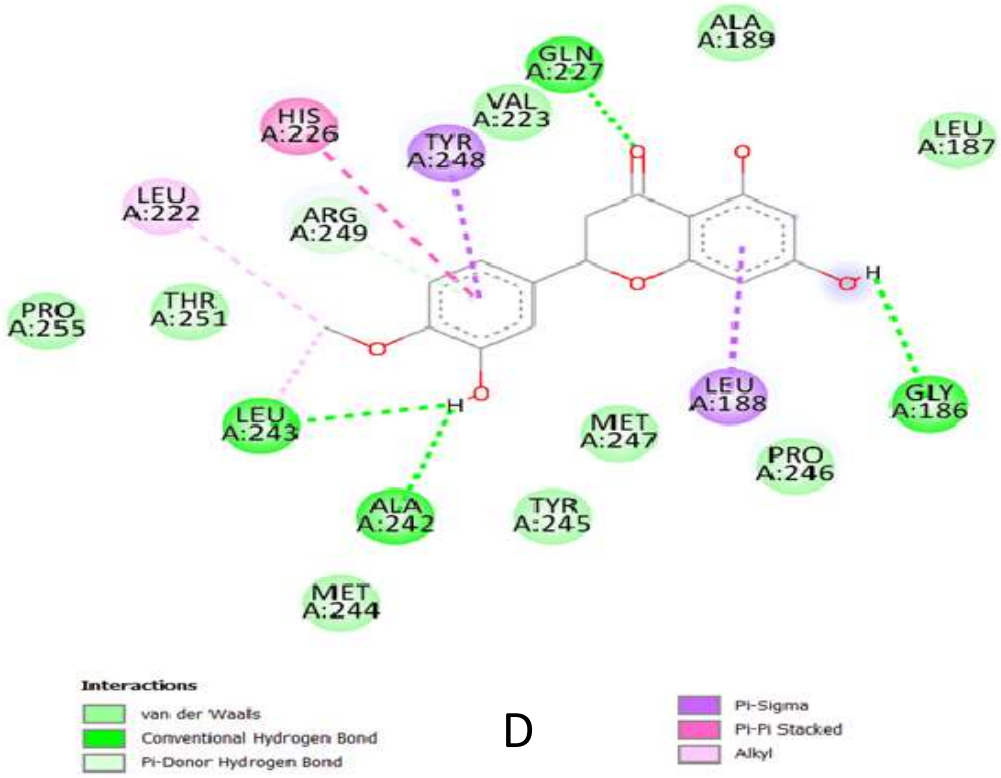
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Interactions
van der Waals
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Pi-Sigma
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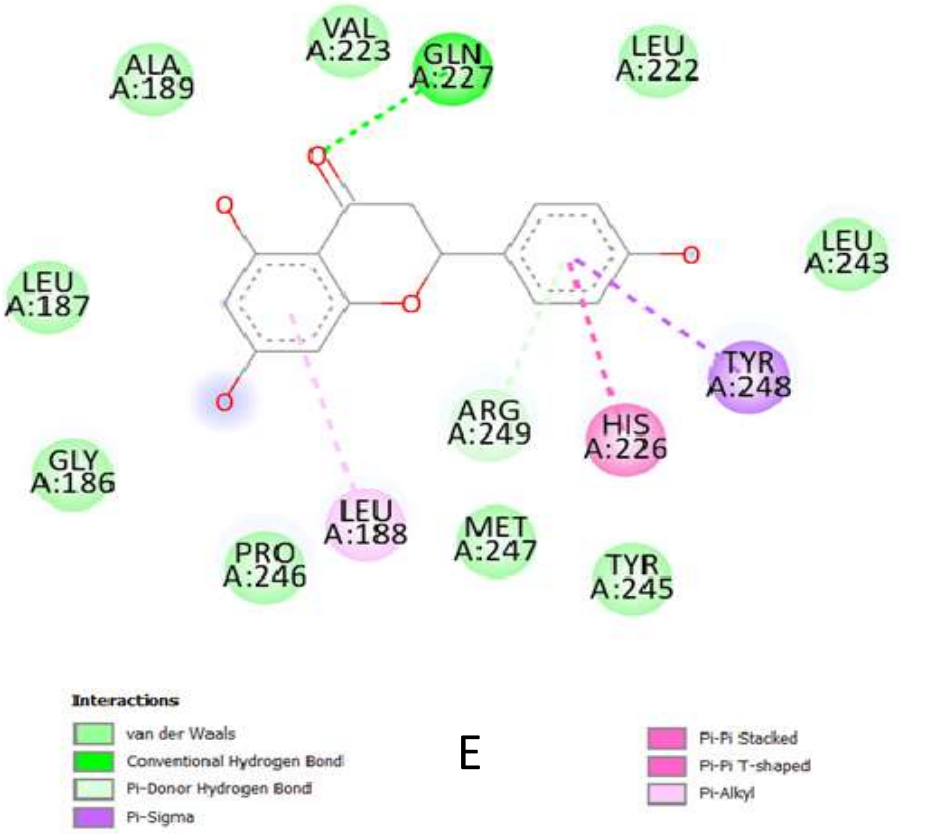
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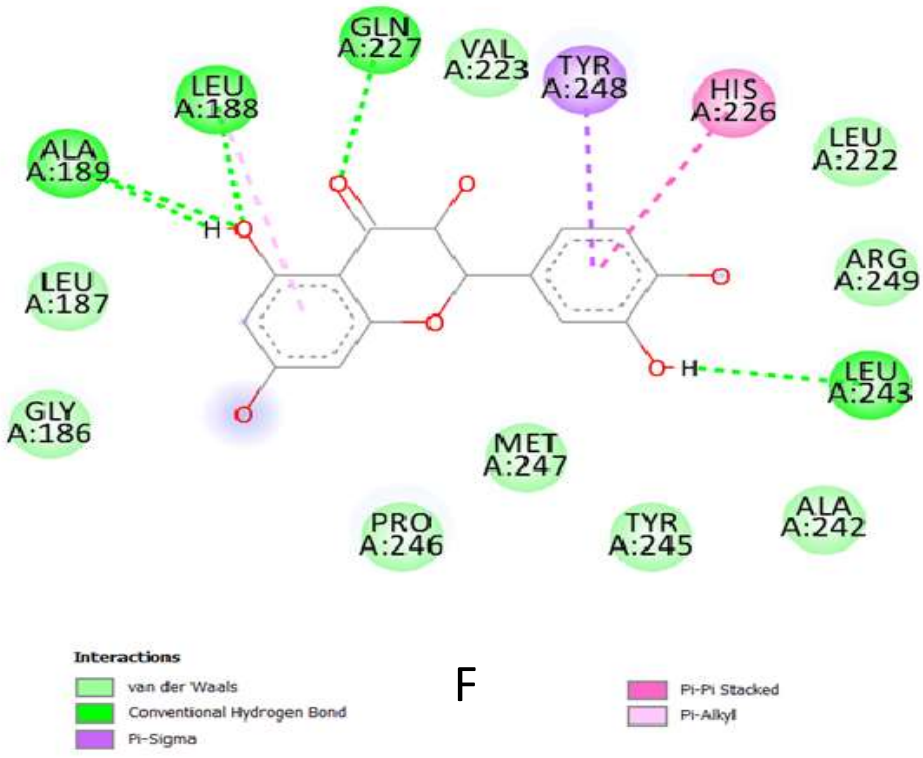
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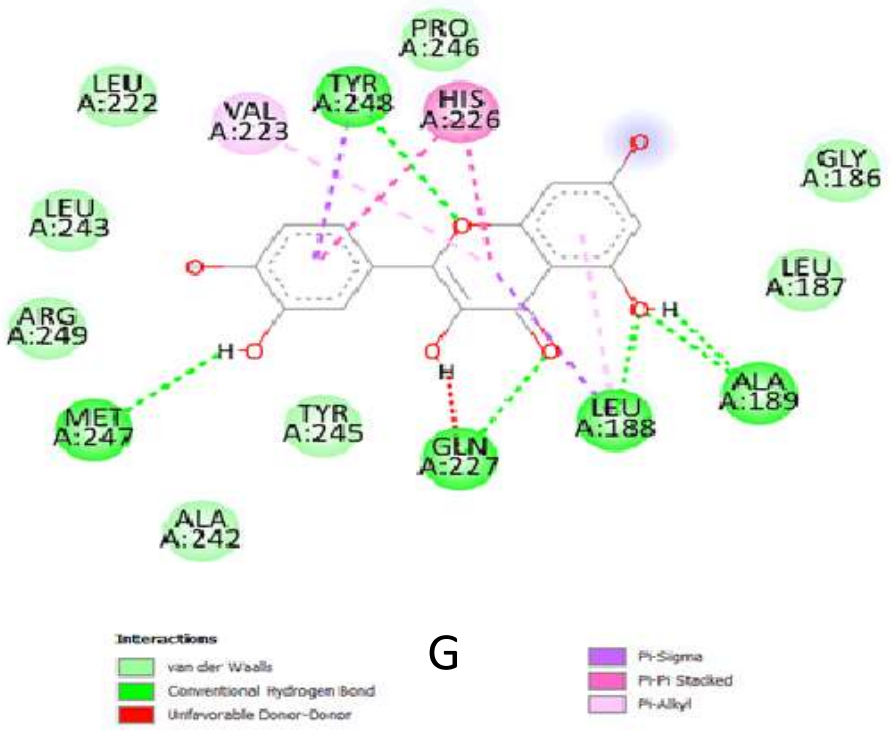
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Pi-Sigma
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Pi-Alkyl

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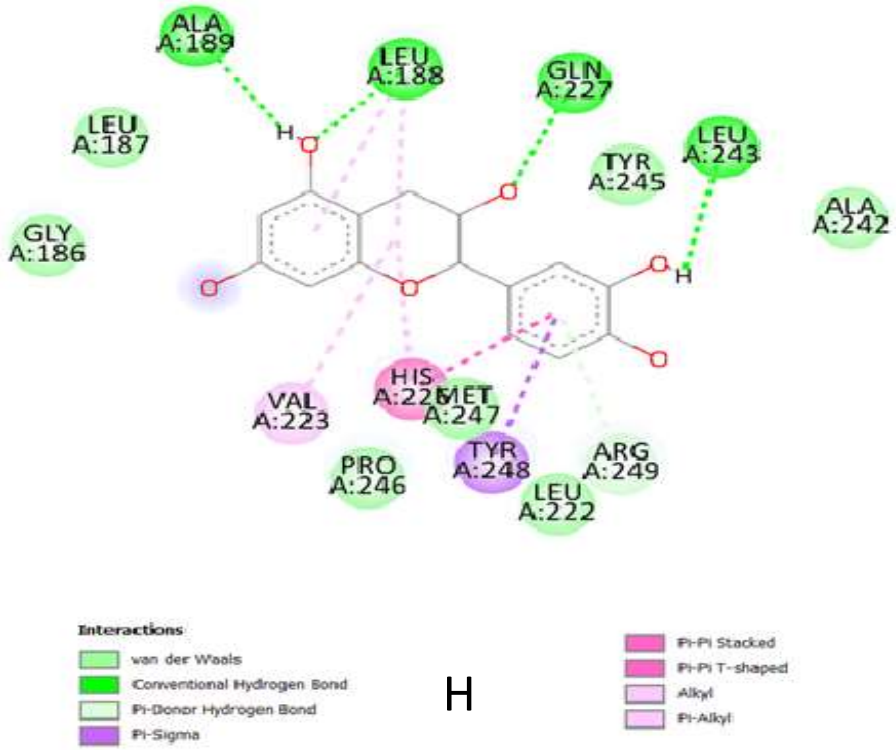
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Pi-Pi T-shaped
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Pi-Alkyl

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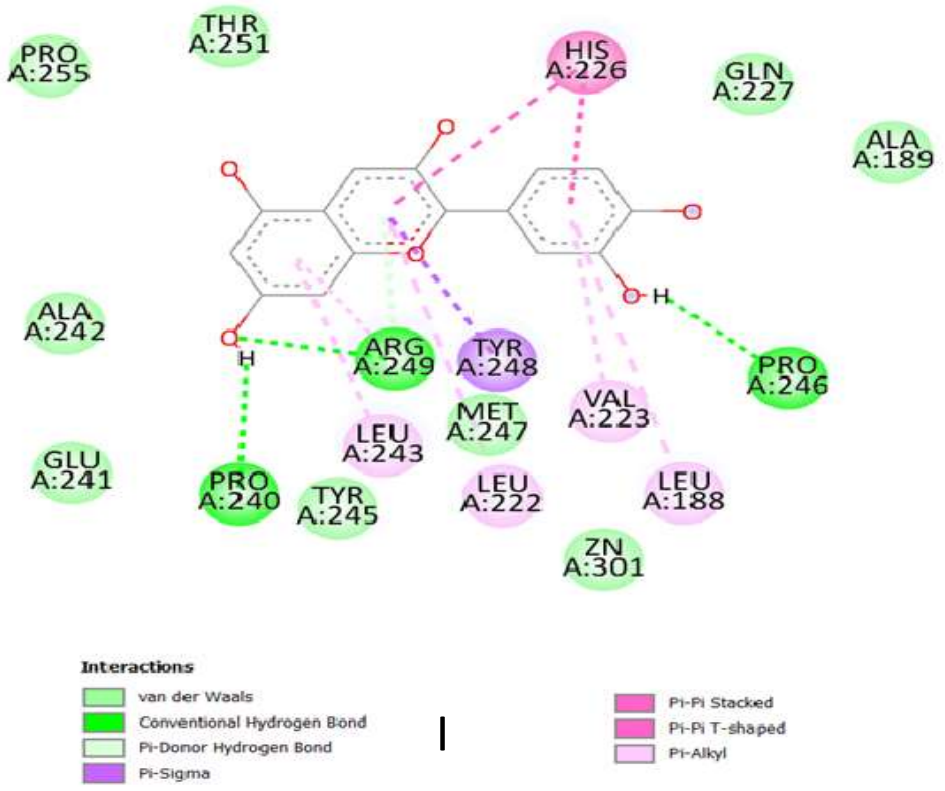
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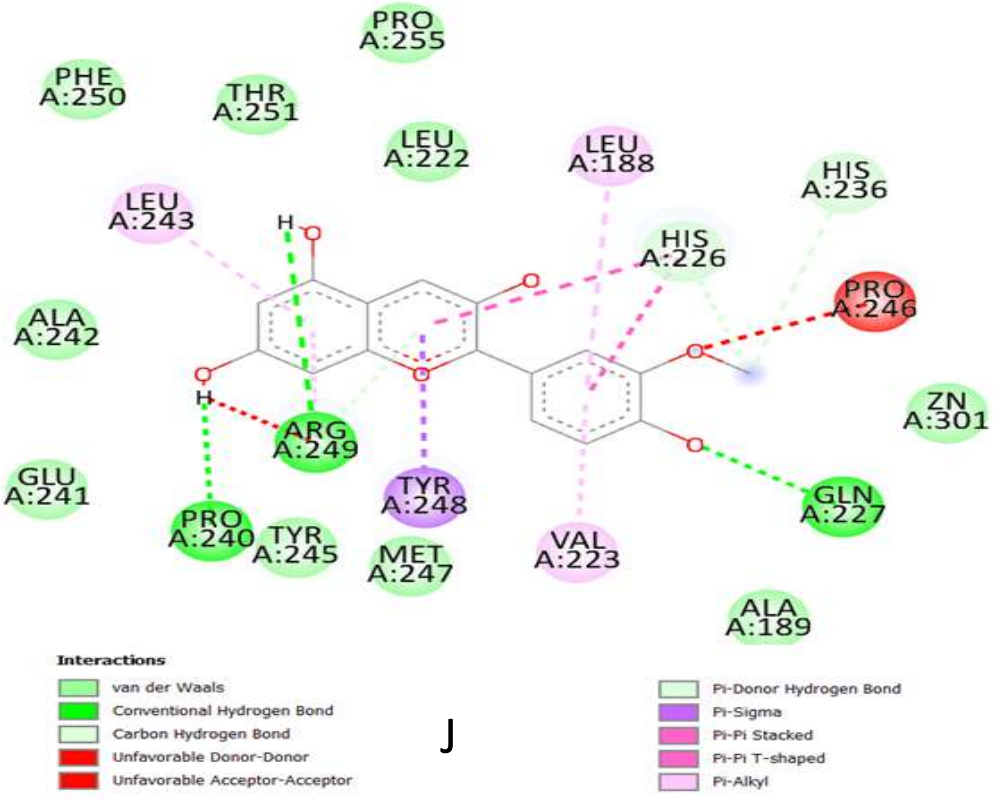
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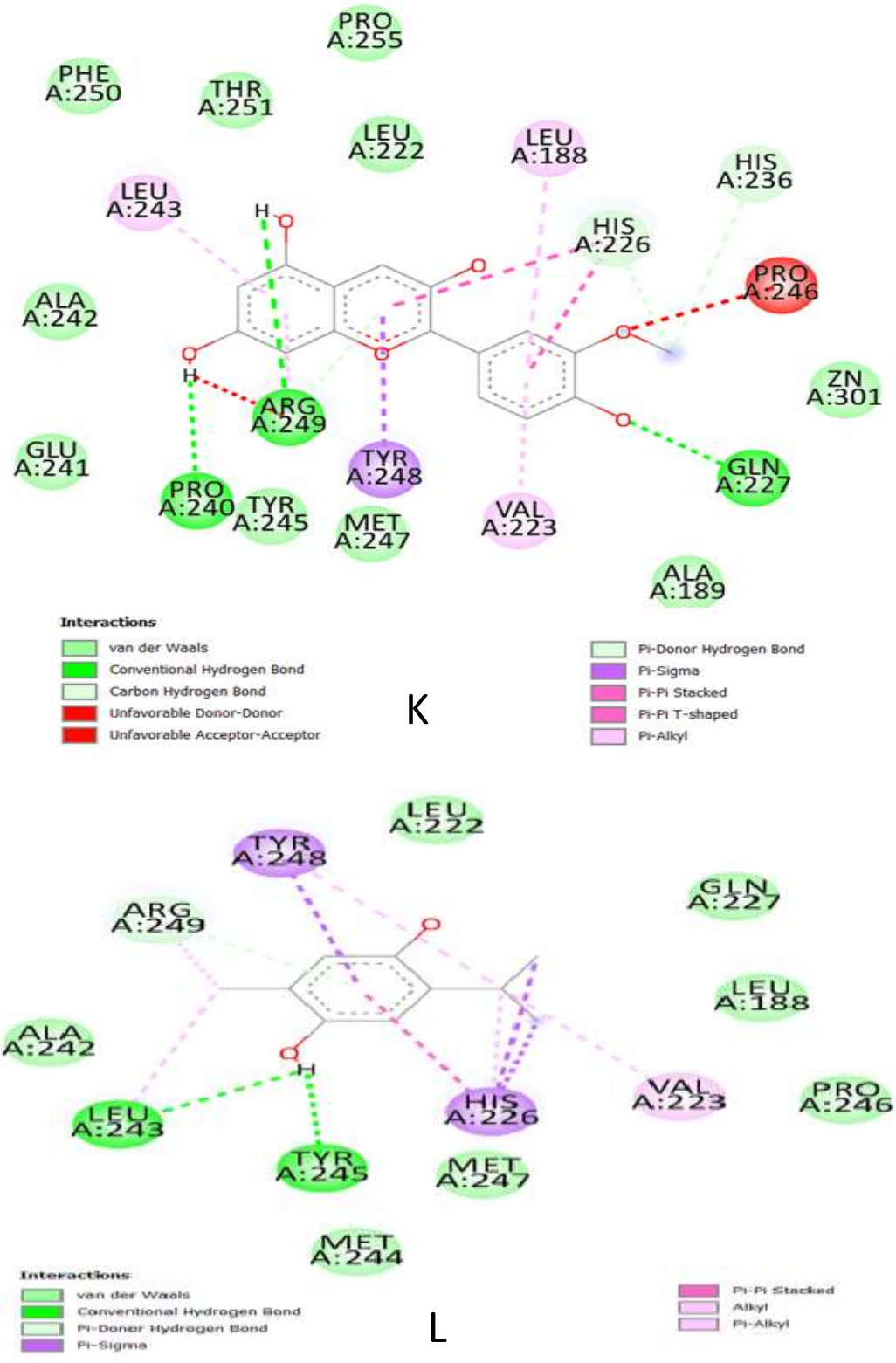


Fig.7: - 2D-Molecular docking surface view between MMP-9 (6ESM) and selected flavonoids: (A) Apigenin, (B) Chrysin, (C) Luteolin, (D) Hesperetin, (E) Naringenin, (F) Taxifolin, (G) Quercetin, (H) Catechin, (I) Cyanidin, (J) Peonidin, (K) Genistein and (L) Thymohydroquinone.

3.3.4 Molecular Docking of Combinations of Flavonoids

The top 10 compounds that showed the best binding energies in individual ligand docking were considered for combination dockings to study their synergistic inhibition effect against MMP-9. All the combination docking conformations showed significantly high binding energies compared to the individual dockings. The two best combinations (Quercetin- Genistein and Luteolin and Genistein) that interacted with the active site residues were docked 100 times independently and the highest value, average values, and standard deviations of the binding energies are presented in (Table 6). The highest binding energy of quercetin and genistein was -15.48 kcal/mol and luteolin and genistein was -15.31 kcal/- mol for MMP-9. The 2D and 3D docking conformations of the two combinations in (Fig. 8). Both the combinations of flavonoids makes many hydrogen bonds with LEU188, ALA189, HIS226, HIS230, ASP235, TYR245, PRO246, and MET247 revealing effective inhibition of MM- P-9 synergistically.

Table 6: -Molecular docking binding energies and amino acids interaction of the two best combinations of flavonoids with MMP-9 (6ESM).

Flavonoid Combination with 6ESM	Amino Acids Interaction	Amino acid with hydrogen bond interaction	Highest Binding Energy (Kcal/mol)
Quercetin-Genistein	HIS230, GLN227, PHE181, ALA189, LEU187, PRO180, TYR179, HIS190, HIS236, MET247, PRO246, HIS226	ALA189, PRO180, PRO246	-15.48
Luteolin and Genistein	PHE192, ALA191, HIS236, HIS230, HIS190, HIS226, GLN227, ALA189, LEU188. ASP235, PRO193,	ASP235, ALA191, ALA189	-15.31

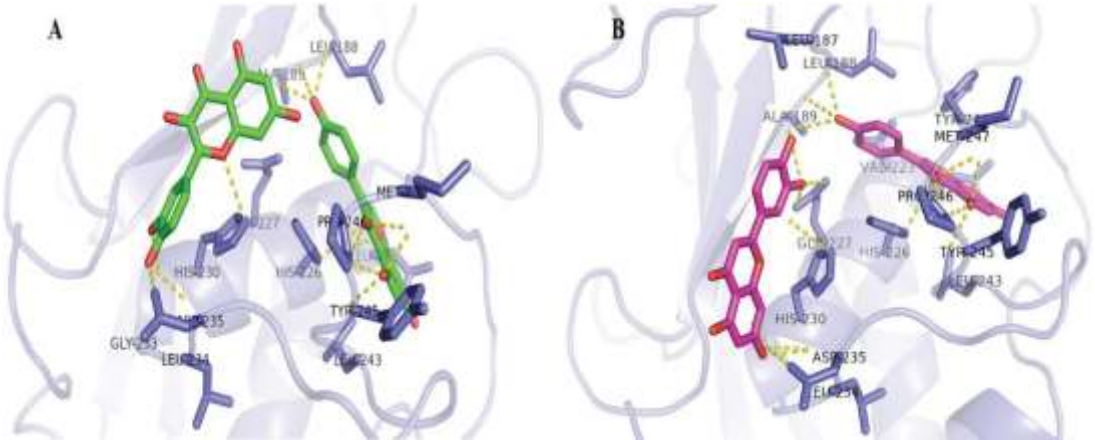


Fig. 8: - 3D Molecular docking conformations of MMP-9 (6ESM) with a combination of (A) Quercetin and Genistein and (B) Luteolin and Genistein

3.4 Conclusion

The present combination analysis of flavonoids for structural inhibition of MMP-9 provided quercetin and luteolin in combination with genistein identified as the potent flavonoid combinations. The PCA investigation of the physiochemical and bioactive properties of flavonoids in turn depicted similarity in physio chemical and biological properties of quercetin and luteolin. Genistein showed a significant binding energy of -9.54 kcal/ mol in individual docking and in combinations with quercetin and luteolin showed highly efficient binding energy values of greater than -15 kcal/mol revealing the synergistic effect of the combinations of flavonoids. In both combinations, the binding orientation of genistein was at the same position displaying its high affinity binding at the respective site containing LEU188, ALA189, HIS226, TYR245, PRO246, and MET247.

CHAPTER – 4

In-Silico Combinatorial inhibition effects analysis of NSAIDs against MMP-9 for the treatment of cancer

4.1 Introduction

Cancer refers unusual division and growth of body cells with the capability to proliferate to distant parts of the body. It is the second major cause of death worldwide. The International Agency for Research on Cancer gave a detailed report on global cancer occurrence and death based on GLOBOCAN (Global Cancer Observatory) 2020 data. According to report about 19.3 million fresh cancer cases identified, and about 10.0 million cancer patients died worldwide in the year 2020. Approximately 2.3 million (11.7 %) new breast cancer cases and 2.2 million (11.4 %) new lung cancer cases were identified in the year 2020. Also, an increase to 28.4 million cancer patients by the year 2040 has been proposed (Sung et al., 2021)

According to the National Cancer Registry Programme (NCRP) Report 2022, India's estimated breast cancer occurrence and prevalence rate was 105.4 per 100000 in females, and the lung cancer rate was 95.6 per 100000 in males. The occurrence of cancer cases in India is known to increase by 12.8 % from the year 2022 to 2025. The Global Cancer Observatory forecasted 2.08 million cancer cases, indicating a rise of 57.5 % from the year 2020 to 2040 for India. The most common body parts prone to cancer are the digestive system, breast, genitals, oral cavity, and respiratory system. Lung cancer is most prominent in males, while breast cancer is in females.

Matrix metalloproteinase 9 (MMP-9) is a component of the family of Gelatinase B, and it is capable of degrading gelatin. It is normally present in the cerebellum, hippocampus, and cerebral cortex (Xiao et al., 2024). The bone marrow is the main site for the synthesis of MMP-9, which is then stored in neutrophils. Further, macrophages are also a dominant originator of MMP-9 (Y. Wang et al., 2024). Upregulation of MMP-9 has promoted the progression of many diseases, such as emphysema in Smad3-null mice. MMP9 overexpression also enhances the invasiveness of the LNCaP cell line of prostate tumor.

MMP-9 (Gelatinase B) has been reported to promote cancers. In lung cancer, MMP-9 is induced by Skp2, a constituent of the E3 ubiquitin ligase. Skp2 has significant function in the induction of p27 degradation; thus, overexpression of Skp2 may cause an increase in p27 proteolysis and encourage cell and tumor invasion and metastasis (Hung et al., 2010). In breast cancer, the overexpression of MMP-9 relates to the expression of transcription factor activator proteins AP-2 and HER2. The overexpression of HER2 and AP-2 is responsible for MMP induction and gelatinase regulation (Pellikainen et al., 2004). Overexpression of MMP-9, therefore, has a strong connection with the extensive range of cancers and their progression, so MMP-9 can be considered as a potential target to develop effective therapies against cancer.

Nonsteroidal anti-inflammatory drugs are frequently used drugs for the treatment of pain, fever, stiffness, and inflammation. Globally, over 300 lakhs people use NSAID per day. Asprin has been used for the last 120 years and is considered the procreator of all NSAIDs**.** Based on chemical structures, COX inhibitory properties, and selectivity, the NSAIDs are classified as non-selective and selective NSAIDs. The non-selective NSAIDs include NSAIDs-carboxylic acid (Asprin, Naproxen, Diclofenac, Ibuprofen, Indomethacin, Ketoprofen, and Flurbiprofen), Oxicams (Piroxicam), preferential COX-2 inhibitors-Carboxamides (Meloxicam), Sulphonanilides (Nimesulide), and Naphthalenes (Nabumetone), while the selective COX-2 inhibitors include diaryl-substituted Pyrazoles/Furanones (Celecoxib, Rofecoxib, Valdecoxib) (Ozleyen et al., 2023). The major mode of action of NSAIDs is inhibition of cyclooxygenase (COX-1 and COX-2)

Wang et al., 2020, studied that flurbiprofen inhibits inflammatory factor expression, multiplication, invasion, and migration of colorectal cancer cells by suppressing the expression of COX2 and MMP-9. The inflammatory factor inhibition is measured by TNF-α, IL-β, and IL-6 levels through ELISA. These factors are decreased in flurbiprofen-treated cells. Moreover, multiplication, invasion, and migration were measured by transwell and wound healing assay with SW620 cells. The western blotting method showed the inhibited expression of MMP-9 in the samples treated with Flurbiprofen (X. Wang et al., 2020). Prasad et al., 2024 studied the protective effects of NSAIDs (Aspirin and Naproxen) in TMPSS2-ERG fusion-driven prostrate tumorigenesis as inhibitory effects in proliferation and inflammation. The effect of

NSAIDs was concerned with the inhibited expression of M-CSF, IL-33, CCL22, CCL12, and CD93, which are tumor-promoting factors; chemerin, Fit-3 ligand, and IGFBP-5, which are growth signaling molecules, and MMP-9, which are stromal alternation proteins (Prasad et al., 2024). Syggelos et al., 2007 investigated the inhibitory effects of NSAIDs on both MMP-2 and MMP-9 by gelatin zymography (Syggelos et al., 2007). Fisher & Demel, 2019 discussed NSAIDs as potential therapeutic agents in overcoming inflammation in intracranial aneurysms (IA) progression. They effectively suppress many inflammatory factors, including nuclear factor-kB and MMPs (MMP-9) which are involved in IA. Various studies have been focused on the downregulation of MMP-9 through NSAIDs for treating cancer and other inflammatory responses (Fisher & Demel, 2019).

Therefore, the effective role of MMP-9 in the development and progression of carcinogenic conditions and the efficient anti-inflammatory properties of the Non-Steroidal Anti-inflammatory drugs (NSAIDs), provide a foundation to the present study for the identification of potential NSAID combinations that display synergistic effects and can inhibit the MMP-9 structurally, using an in-silico approach, to provide a high potential treatment against cancers.

4.2 Methodology

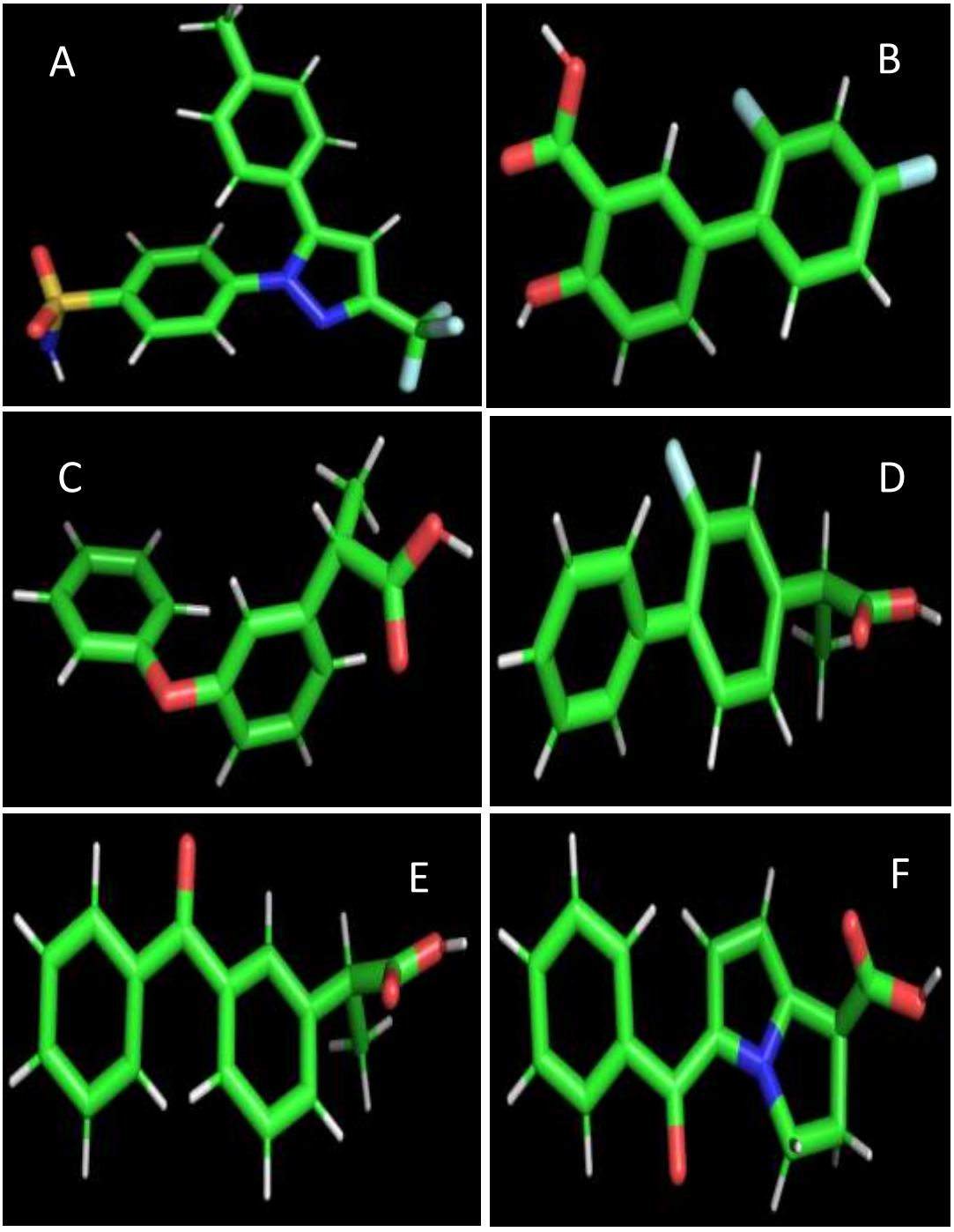
4.2.1 ADMET Analysis and Principal Component Analysis (PCA)

Previous studies and anti-inflammatory properties of non-steroidal anti-inflammatory drugs (NSAIDs) focused on the selection of Diflunisal, Fenoprofen, Flurbiprofen, Ketoprofen, Ketorolac, Nabumetone, Naproxen, Oxaprozin, Piroxicam, and Celecoxib (Table 7 and Fig 9) for identification of their possibilities as MMP-9 inhibitor. The structures of the all 10 selected NSAIDs were drawn, using ChemDraw Ultra Version 12.0 for the stereochemistry, and converted into SMILES format. The physiochemical properties of these NSAIDs were evaluated using SwissADME (Daina et al., 2017).

The analysis of toxicity was performed using Pro Tox II while the bioactivity was analyzed in-silico by Molinspiration (https://www.molinspiration.com/) web servers

respectively (Banerjee et al., 2018). The Origin 2023b was used for generating a chord diagram for comparison of different NSAID properties.

The Minitab trial version 2021 was utilized for conducting the PCA which evaluates the connection between the bioactivity, physiochemical properties, and toxicity of the selected NSAIDs.



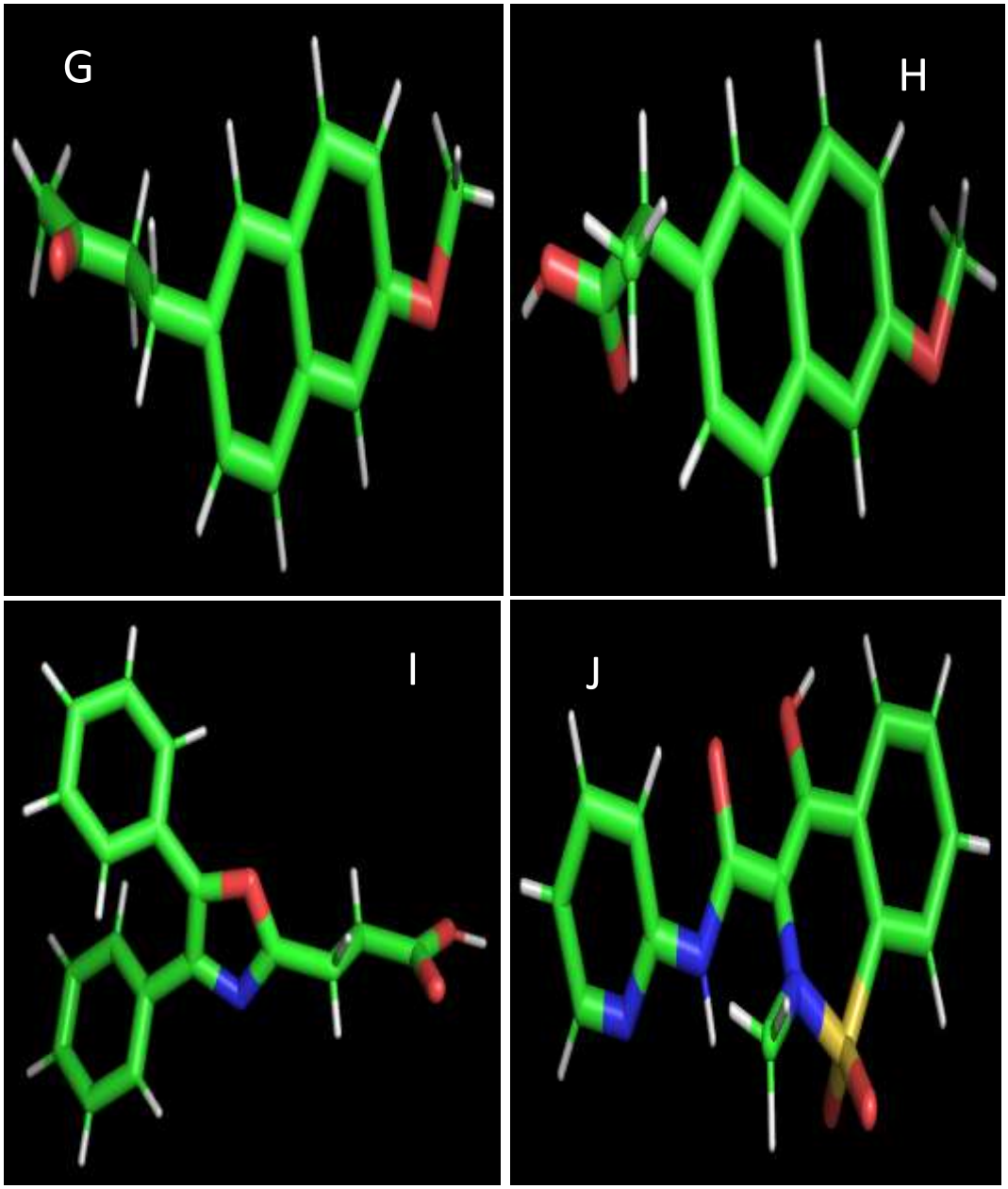


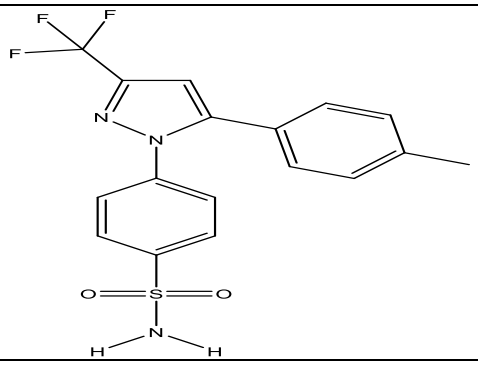
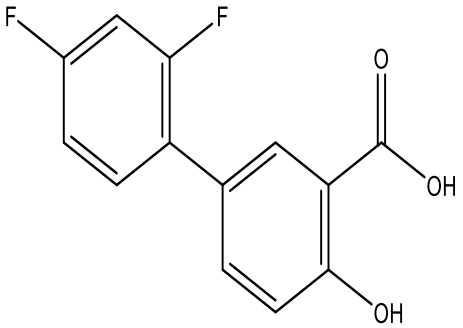
Fig. 9: - 3D View NSAIDs: (A) Celecoxib, (B) Diflunisal, (C) Fenoprofen, (D) Flurbiproxen, (E) Ketoprofen, (F) Ketorolac, (G) Nabumetone, (H) Naproxen, (I) Oxaprozin, (J) Piroxicam

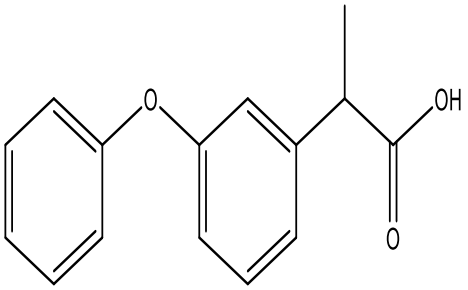
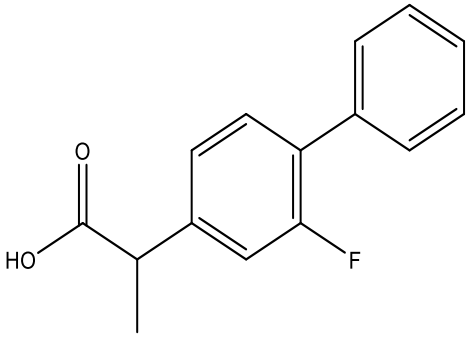
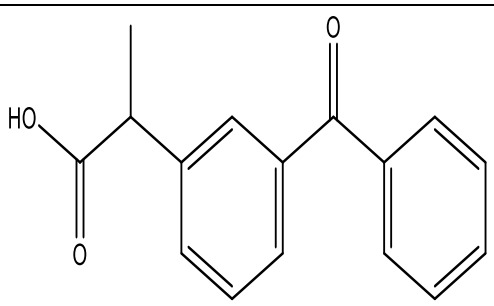
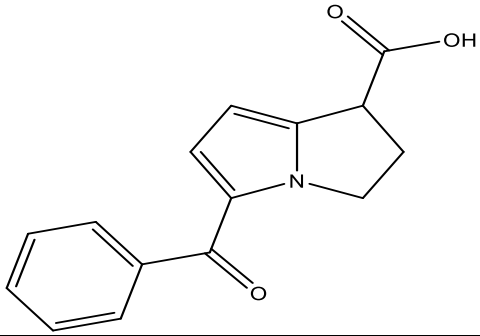
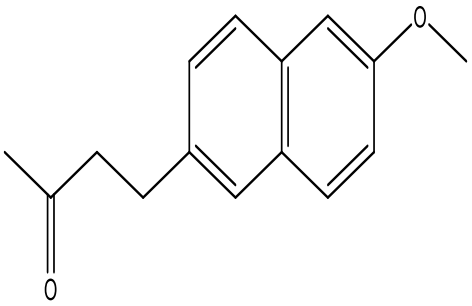
4.2.2 Molecular Docking

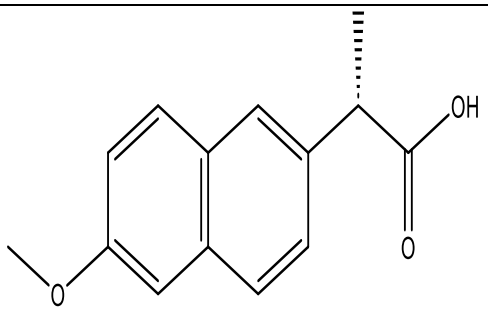
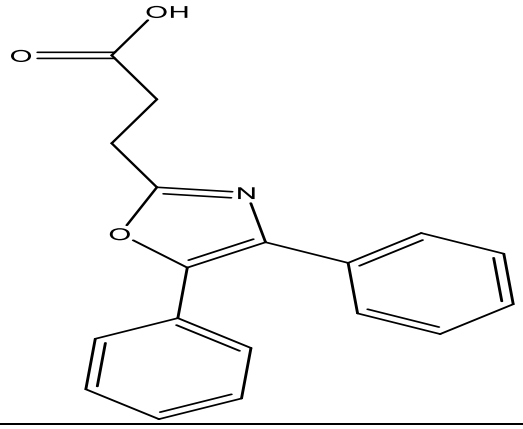
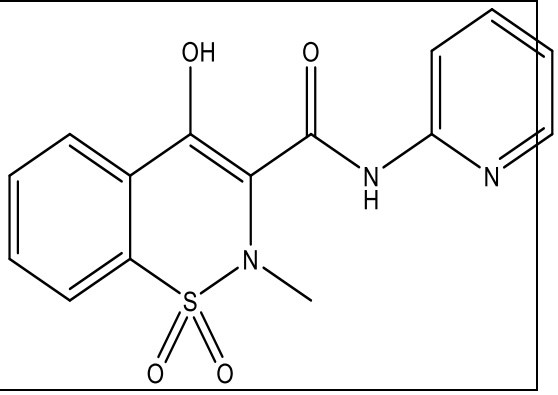
The crystal structure of MMP-9 was downloaded from RCSB-PDB, which had PDB ID 6ESM. The structures of selected non-steroidal anti-inflammatory drugs (NSAIDs) in 3D-conformations were generated by online smile translator tool (https://cactus.nci.nih.gov/translate/). Molecular docking was performed by

AutoDock Tools 1.5.6 (https://autodock.scripps.edu/), individually and in combinations at the active site having coordination of 3 histidine (His 226, His 230, His 236, and Zn. For combination docking, two NSAIDs were considered together in pdbqt format to perform docking. The Kollman charges of -75.265 atomic units were added to the MMP-9. The grid size X = 12.165, Y= 15.184, Z= 18.128, grid center: X = 1.582, Y = 50.36, Z = 19.54; and grid spacing of 0.33 Å was used for docking. The population size =150, the number of evaluations =25,00000, and the number of generations =27,000 were used in the Lamarckian Genetic Algorithm in docking. The crossover and gene mutation rates were 0.8 and 0.02 respectively. The docking binding energies and docking interactions of both individual and combination dockings were analyzed to study the individual and synergistic effect of molecules and the images of best conformations were generated using PyMol (https://www.pymol.org/).

Table 7: - Chemical structure and Pub Chem CID of considered NSAIDs.

S. No.	NSAIDs Name	Pub Chem CID	Chemical Structure
1	CELECOXIB	2662	
2	DIFLUNISAL	3059	

3	FENOPROFEN	3342	
4	FLURBIPROFEN	3394	
5	KETOPROFEN	3825	
6	KETOROLAC	3826	
7	NABUMETONE	4409	

8	NAPROXEN	156391	
9	OXAPROZIN	4614	
10	PIROXICAM	54676228	

4.3 Results

MMP-9 has been established as an effective cancer target due to its overexpression in different types of cancers. In this study, different NSAIDs have been selected based on their chemical and drug-like properties and that have potential to inhibit MMP-9 using molecular docking. The 10 selected NSAIDs effective candidates are Celecoxib, Diflunisal, Fenoprofen, Flurbiprofen, Ketoprofen, Ketorolac, Nabumetone, Naproxen, Oxaprozin, and Piroxicam (Table 7) and the PDB ID of 6ESM was used for 3D structure of MMP-9.

4.3.1 ADMET and PCA analysis

The ADMET analysis was done by Swiss ADME for all the selected NSAIDs. The predicted LD50 values present the lethal median dose of substance required to kill 50% of the test animals. These values ranged from 49mg/kg to 3880mg/kg for the considered NSAIDs, and showed the diverse nature of the selected NSAIDs (Table 9). Lipinski's rule of 5 is considered to assess the drug-likeness of molecules, and molecules having molecular weight ≤ 500 Daltons, LogP ≤ 5, hydrogen bond donors < 5, and hydrogen bond acceptors < 10 are considered to have effective drug-like properties. The analysis based on Lipinski's rule of 5 to evaluate drug-likeness proved that all the ligands follow these rules with no violation, proving that all NSAIDs have high drug-like properties (Table 9). Bioactivity scores showed negative score values for the ability of the considered NSAIDs to convey inhibition of common off-targets or toxic targets (Table 8).

Table 8: - Bioactivity scores of the considered NSAIDs estimated by the Molinspiration online server.

S. No.	Ligands	GPCR ligand	Ion channel modulator	Kinase Inhibitor	Nuclear Receptor Ligand	Protease Inhibitor	Enzyme Inhibitor
1	CELECOXIB	-0.06	-0.27	0.01	0.28	-0.06	0.17
2	DIFLUNISAL	0.01	0.15	0.05	0.26	-0.14	0.22
3	FENOPROFEN	-0.02	0.02	-0.26	0.29	-0.27	0.20
4	FLURBIPROFEN	0.09	0.20	-0.12	0.30	-0.03	0.28
5	KETOPROFEN	0.09	0.07	-0.15	0.39	-0.09	0.27
6	KETOROLAC	0.29	-0.04	-0.09	-0.03	-0.29	0.62
7	NABUMETONE	-0.26	-0.09	-0.70	-0.25	-0.33	0.08
8	NAPROXEN	-0.11	-0.06	-0.38	0.14	-0.26	0.15
9	OXAPROZIN	0.27	0.05	0.06	0.40	-0.16	0.32
10	PIROXICAM	-0.42	-0.57	-0.50	-0.73	-0.04	0.18

Principal Component Analysis is a multivariate technique that correlate information from a number of observed variables related to a subject into a smaller number of variables. It reduces a large dataset of variable to extract essential features known as principal components. Variance indicates the amount of variability of the variables (Greenacre et al., 2022). PCA was performed for the compounds to study the variance and the association among the compounds based on their ADMET properties. The first two components generated by the analysis define the 80.8% variance of the data. The contribution of the top ten principal components in defining explained variance is presented in the Scree Plot (Figure 10). Close association among naproxen, flurbiprofen, ketorolac, and ketoprofen was observed on the basis of ADMET properties in the score plot, where the scores of these molecules lie in the same quadrant and are closely linked to each other (Figure 10). The loading plot displays the association among the variables or the ADMET properties selected for analysis (Figure 10). Among the physicochemical properties of the NSAIDs, molecular refractivity, aromatic heavy atoms, heavy atoms, molecular weight, hydrogen bond acceptors, and total polar surface area (TPSA) were found to be associated with the first principal component, while the number of rotatable bonds was associated with the second component. The LD50 values were positively associated with the second component. The association was observed between the number of rotatable bonds and LD50 values. The biplot compiles both score and loading plots and defines the association of compounds with different properties as well as with first and second components (Figure 10). The compounds fenoprofen, ketoprofen, ketorolac, flurbiprofen, and naproxen were observed to have similar bioavailability scores and thus were closely arranged in the biplot. Nabumetone, with the least TPSA, significant bioavailability, and extremely high LD50 of 3880mg/kg was observed to be different among all the selected NSAIDs. Oxaprozin and piroxicam were distantly associated to the each other on the basis of bioavailability. Still, they were found to have a positive association with the first component due to mild similarity in physicochemical properties. This analysis proves that a diverse variety of NSAIDs have been selected to examine the for identification potential compounds.

Table 9: - ADMET Properties of selected NSAIDs were analyzed by using Swiss ADMET

Molecule	MW	Heavy atoms	Aromatic heavy atoms	Rotatable bonds	H-bond acceptor	H-bond donor	M R	TPSA	Log P	Lipinski violations	Ghose violations	Veber violations	Bio availability Score	Lead Likeness violations	LD50 (mg / Kg)
Celecoxib	381.37	26	17	4	7	1	89.96	86.36	3.4	0	1	0	0.55	1	1400
Diflunisal	250.2	18	12	2	5	2	60.78	57.53	3.27	0	0	0	0.85	1	392
Fenoprofen	242.27	18	12	4	3	1	69.31	46.53	3	0	0	0	0.85	1	800
Flurbiprofen	244.26	18	12	3	3	1	68.19	37.3	3.59	0	0	0	0.85	2	117
Ketoprofen	254.28	19	12	4	3	1	72.67	54.37	2.84	0	0	0	0.85	0	49
Ketorolac	255.27	19	11	3	3	1	69.81	59.3	2.05	0	0	0	0.85	0	189
Nabumetone	228.29	17	10	4	2	0	70.03	26.3	3.23	0	0	0	0.55	1	388
Naproxen	230.26	17	10	3	3	1	66.79	46.53	2.76	0	0	0	0.85	1	248
Oxaprozin	293.32	22	17	5	4	1	83.73	63.33	3.4	0	0	0	0.85	1	1210
Piroxicam	331.35	23	12	3	5	2	87.52	107.98	1.38	0	0	0	0.56	0	216

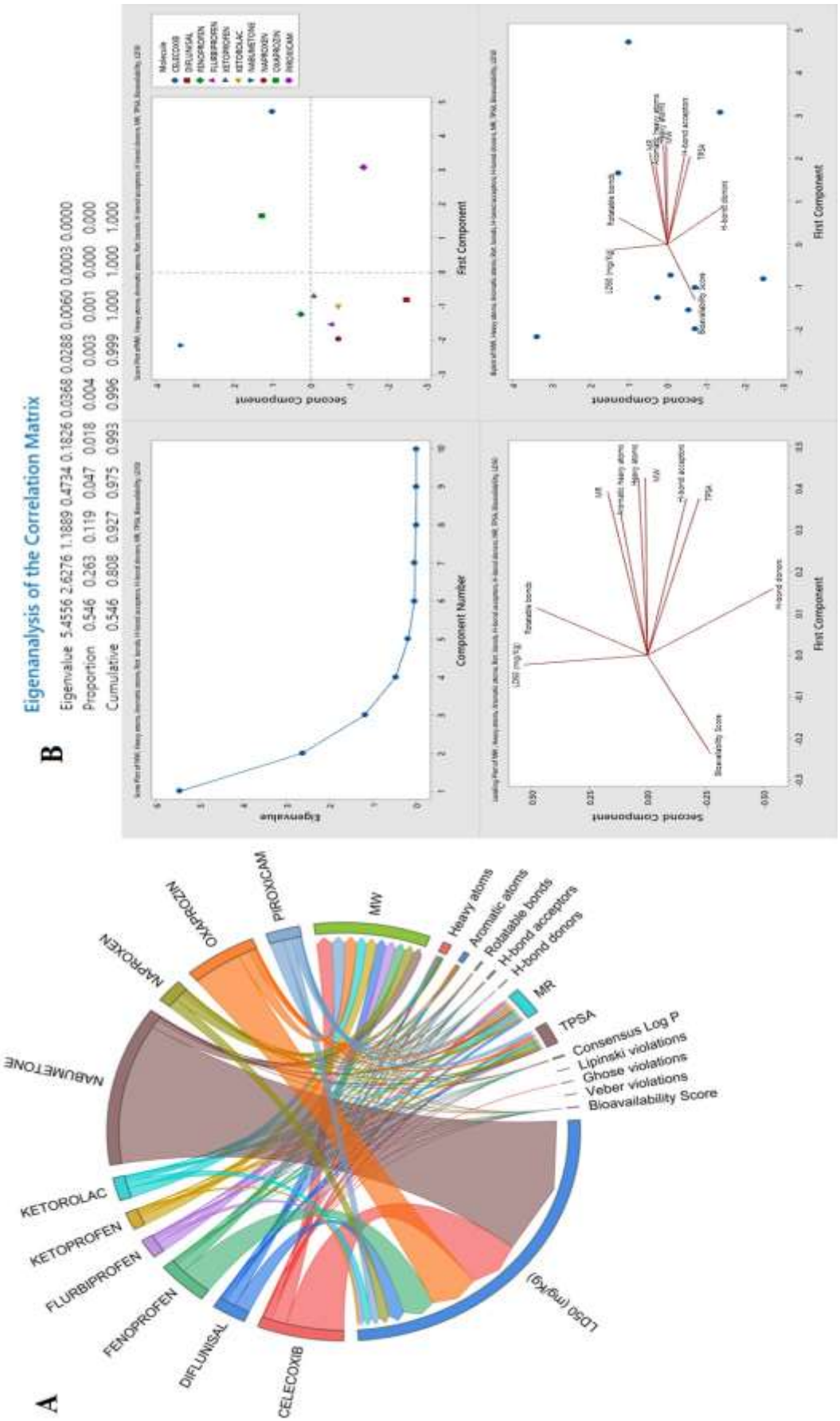
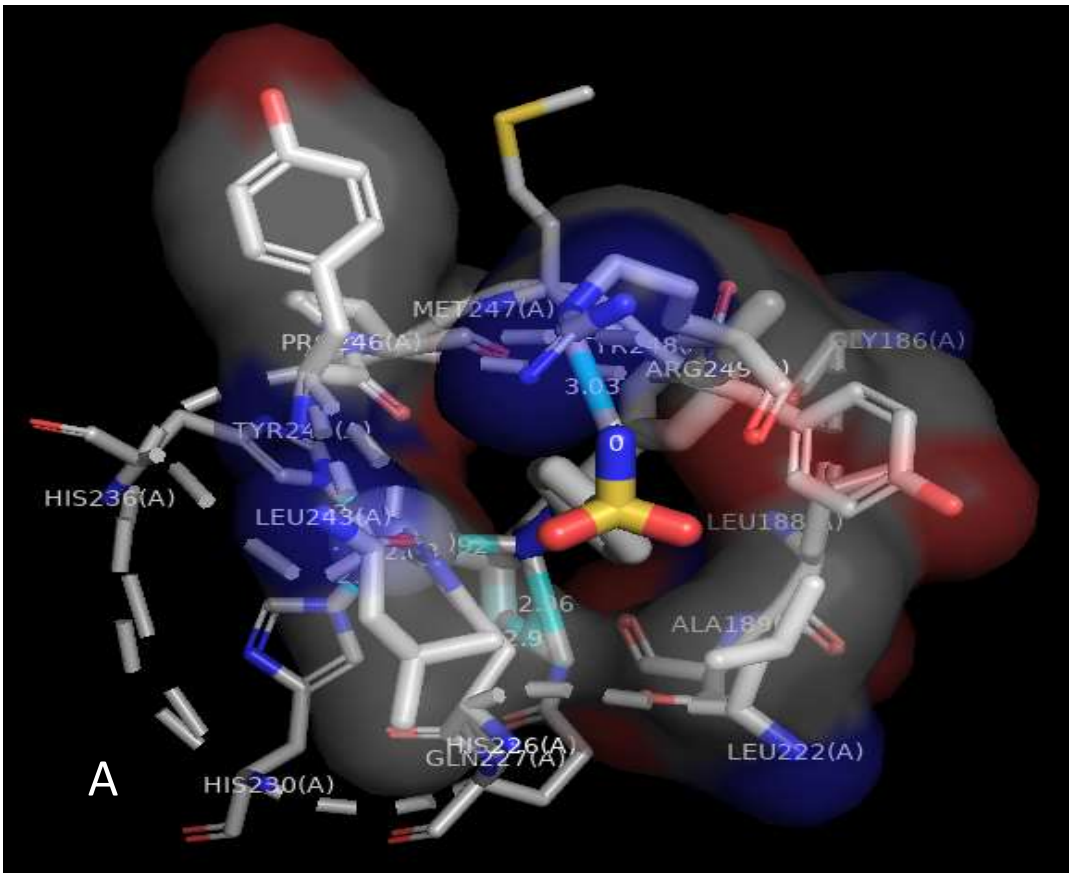
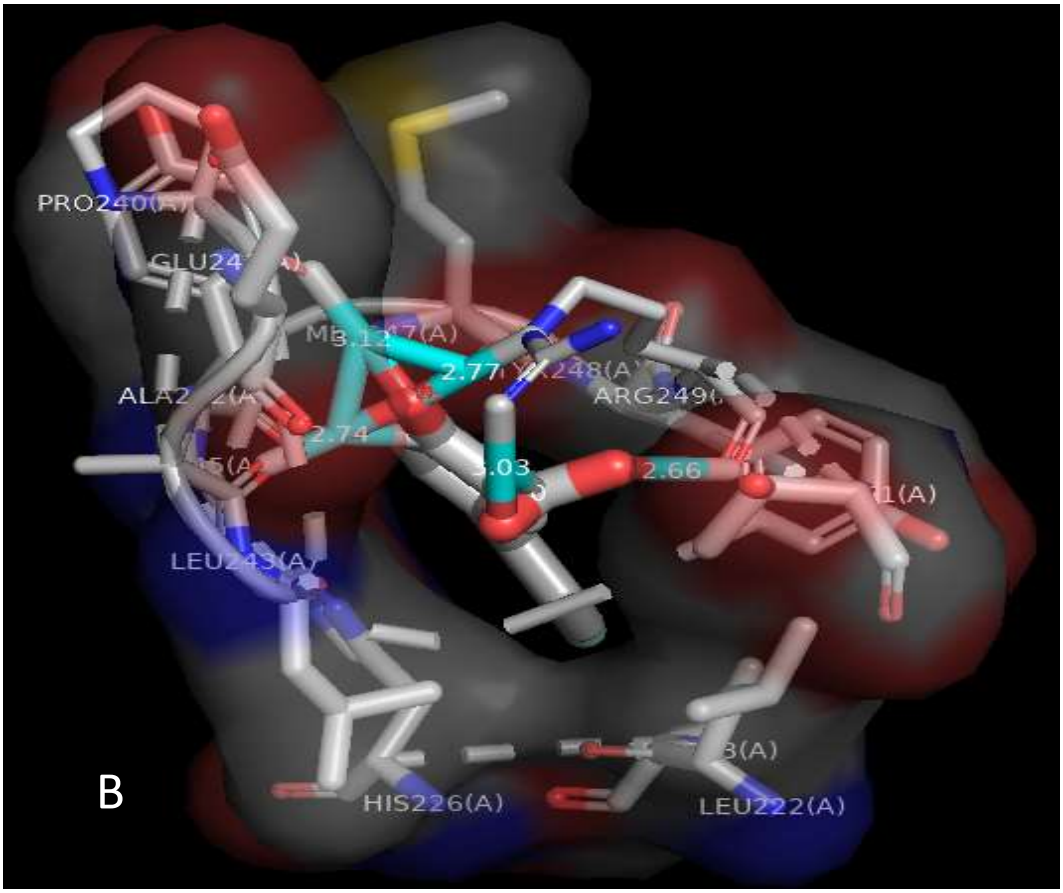


Fig.10: - (A). Chord Diagram showing ADMET properties of the selected 10 NSAIDs (B). Principle Component Analysis Eigen Value Correlation Matix, Scree Plots, Loading Plots, and Biplots.

4.3.2 Molecular Docking of Individual NSAIDs

The docking of MMP-9 with the selected NSAIDs was completed to examine the high-affinity inhibitor. The highest negative binding energies of -12.98 and -12.98 kcal/mol were obtained for Oxaprozin and Piroxicam with MMP-9. The binding energies of other NSAIDs were significant ranging from -12.42 to -11.31 kcal/mol (Table 10). The interaction analysis showed that all the NSAIDs formed significant hydrogen bonding with MMP-9, containing the three histidine-Zn coordination complexes, which mediates the catalysis (Fig. 12 and 13). Flurbiprofen having a binding energy of -12.56 kcal/mol, was observed to form a hydrogen bond with His226 of this coordination complex. The sulfonamide group celecoxib formed four hydrogen bonds at the MMP-9 active site residues with backbone atoms of Val223, Leu243, Tyr248 and Leu222. The diflunisal formed 6 hydrogen bonds with the Met247, Pro246, Tyr245, Leu243, Ala242, and Arg249. Oxaprozin and Piroxicam with the highest binding affinities formed 3 and 5 hydrogen bonds with Gln227, Ala191, and His190 and, Met247, Tyr248, Pro246, His190, and Leu187, respectively.

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ARG249(A)
HIS226(A)
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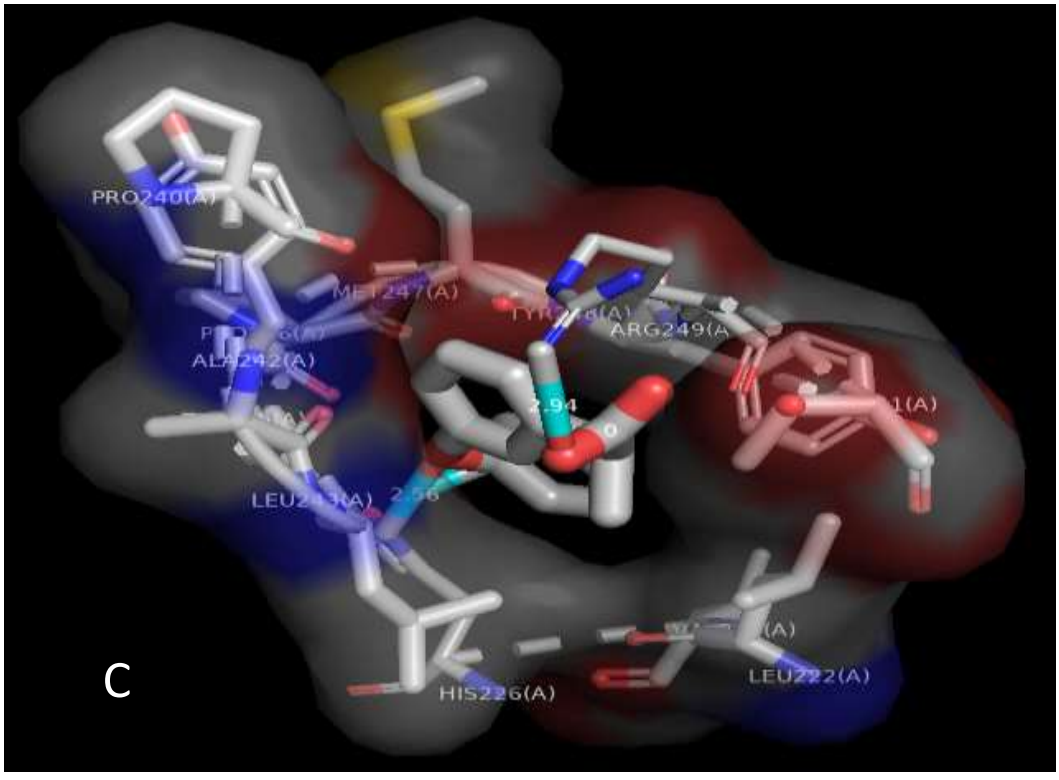
2.66
B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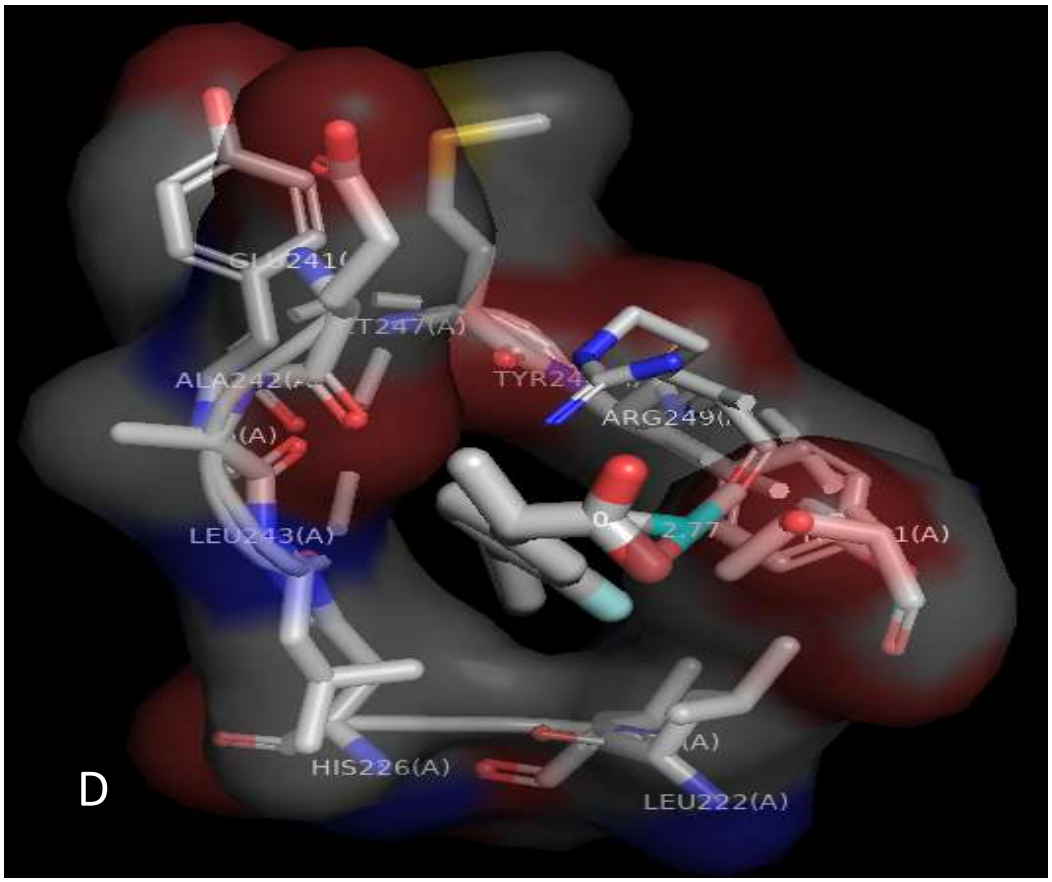
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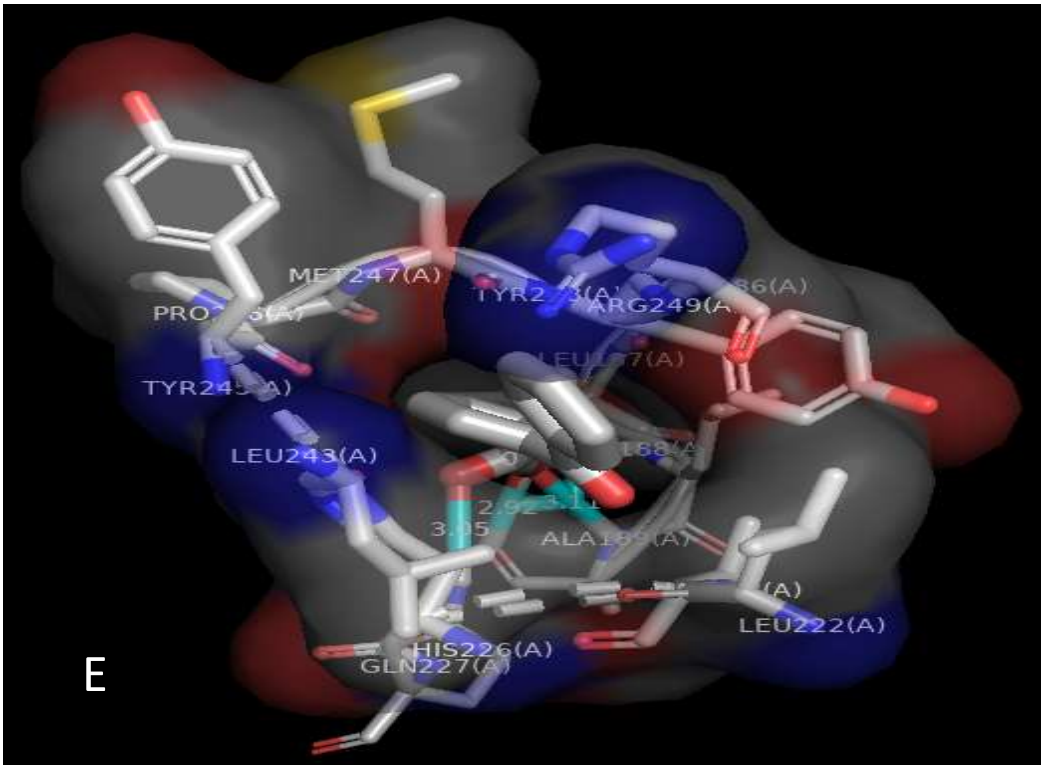
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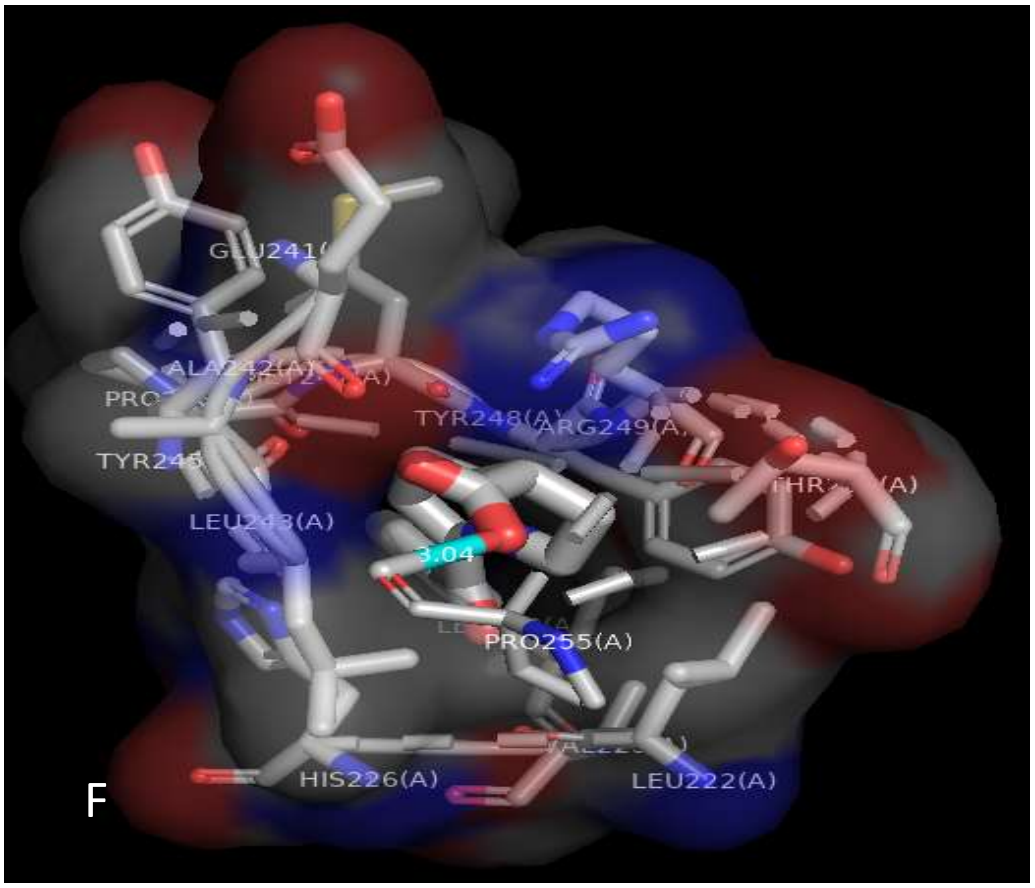
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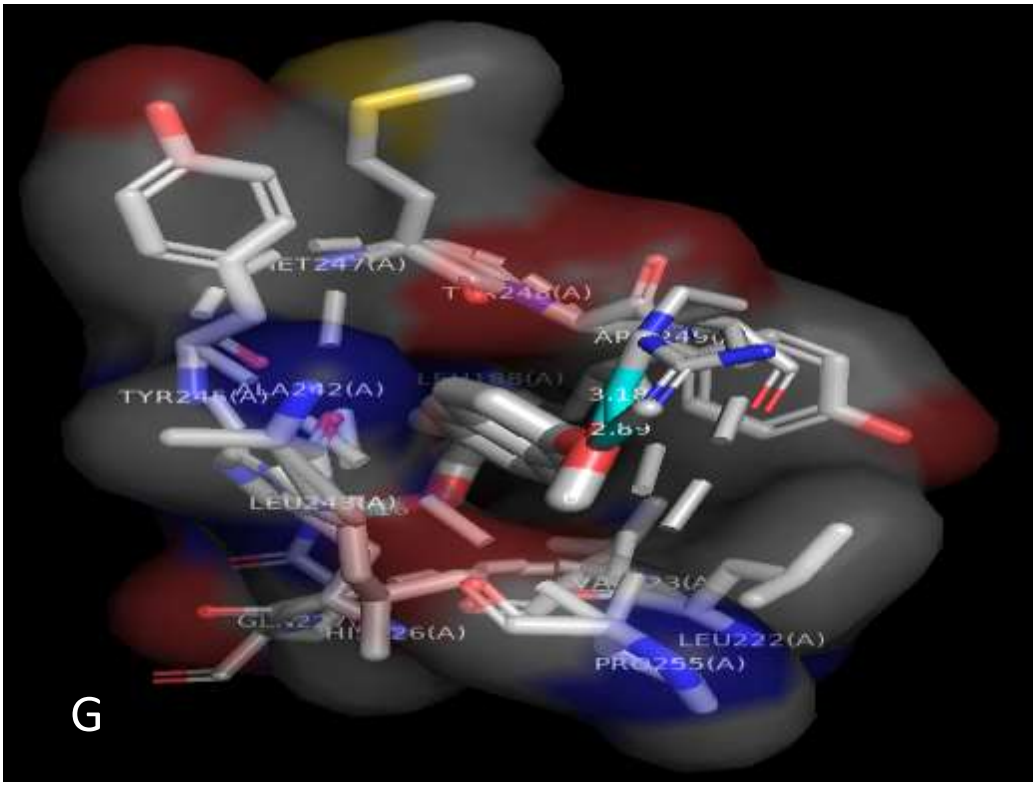
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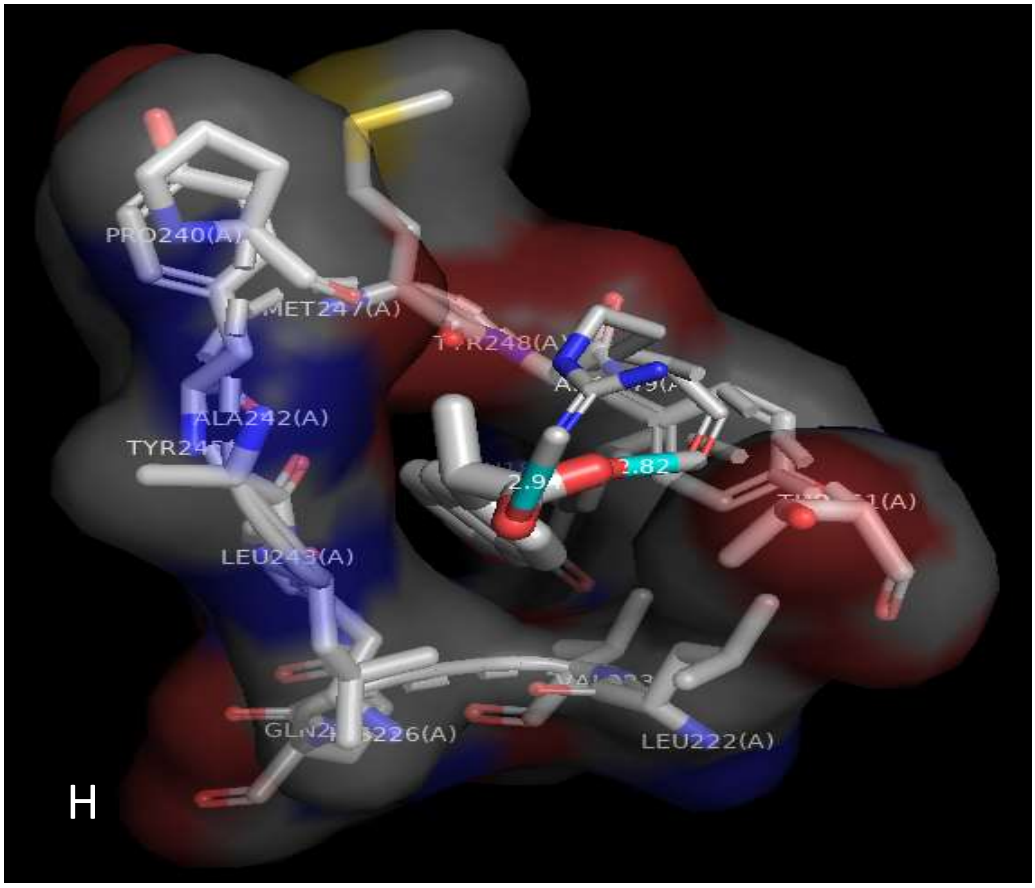
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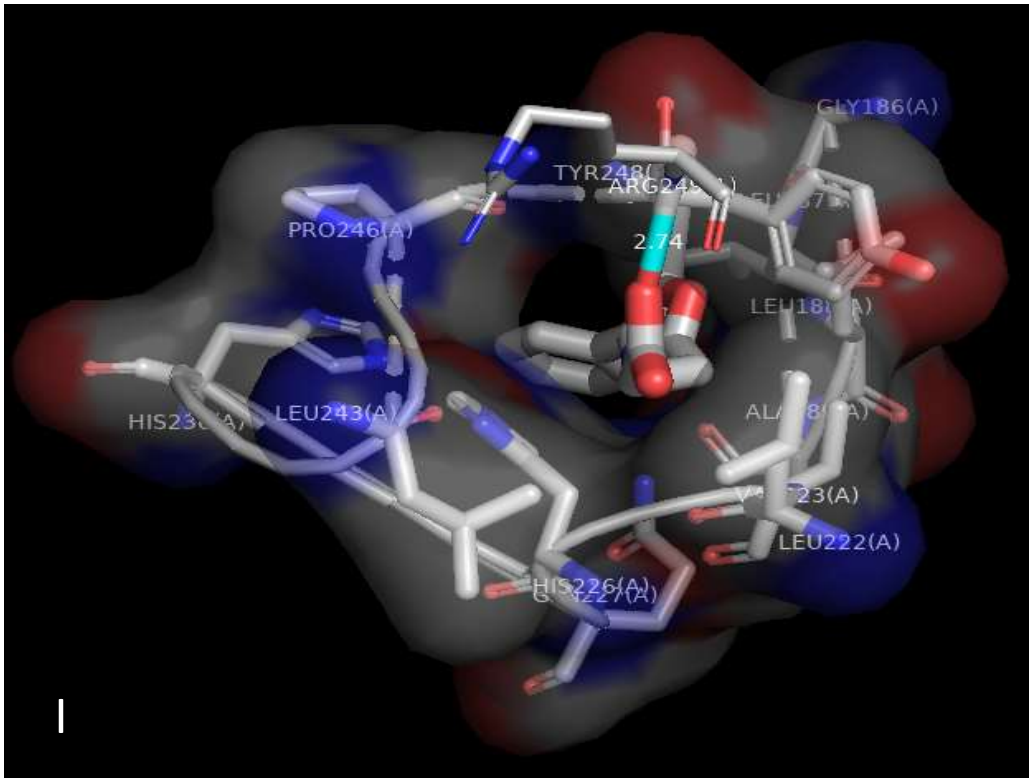
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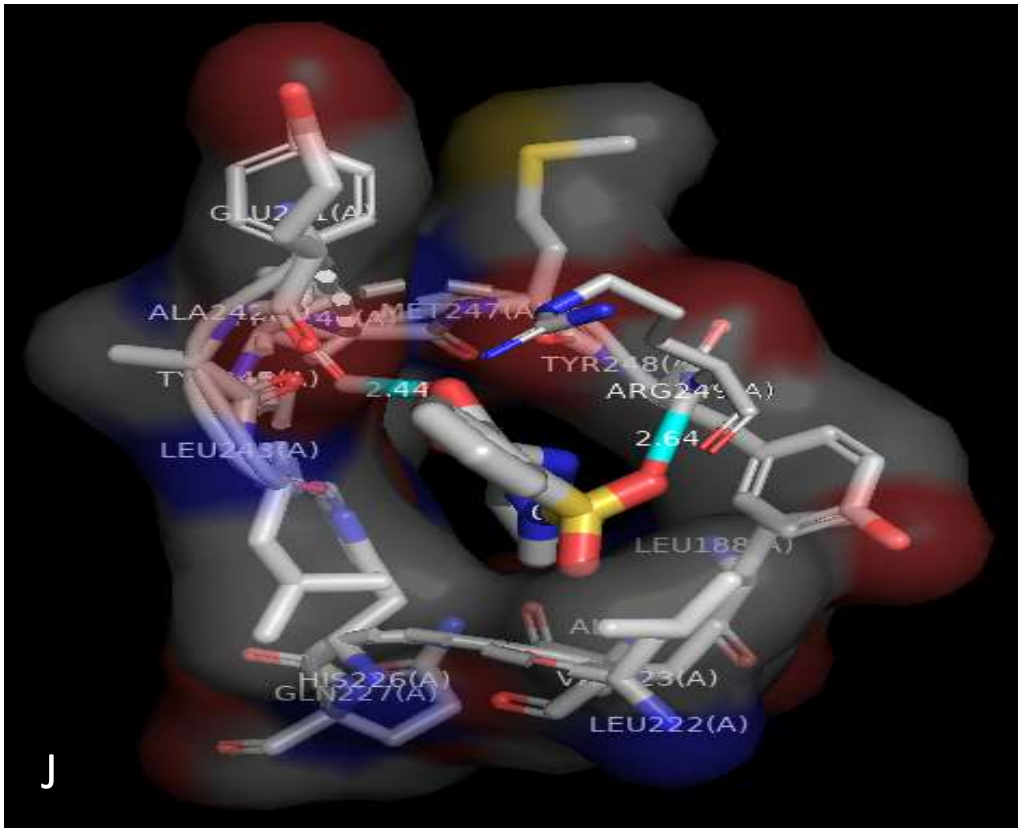
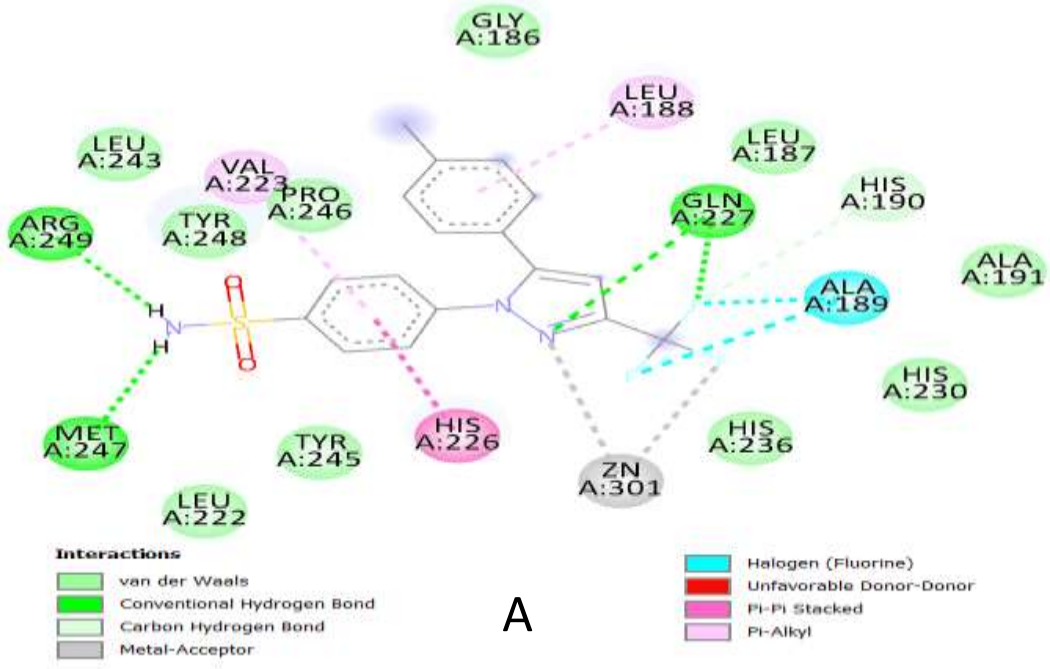
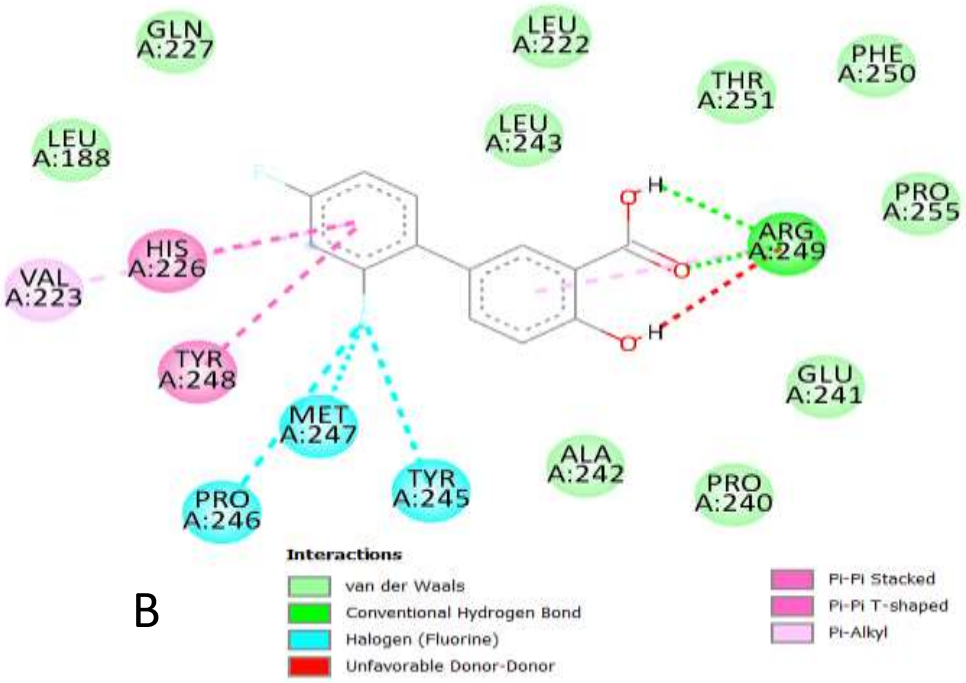


Fig. 11: - 3-D Molecular Docking View of NSAID with MMP-9 (6ESM)-(A) Celecoxib, (B) Diflunisal, (C) Fenoprofen, (D) Flurbiprofen (E) Ketoprofen (F) Ketorolac, (G) Nabumetone, (H) Naproxen, (I) Oxaprozin (J) Piroxicam.

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GLU A:241
MET A:247
ALA A:242
TYR A:245
PRO A:240
PRO A:246
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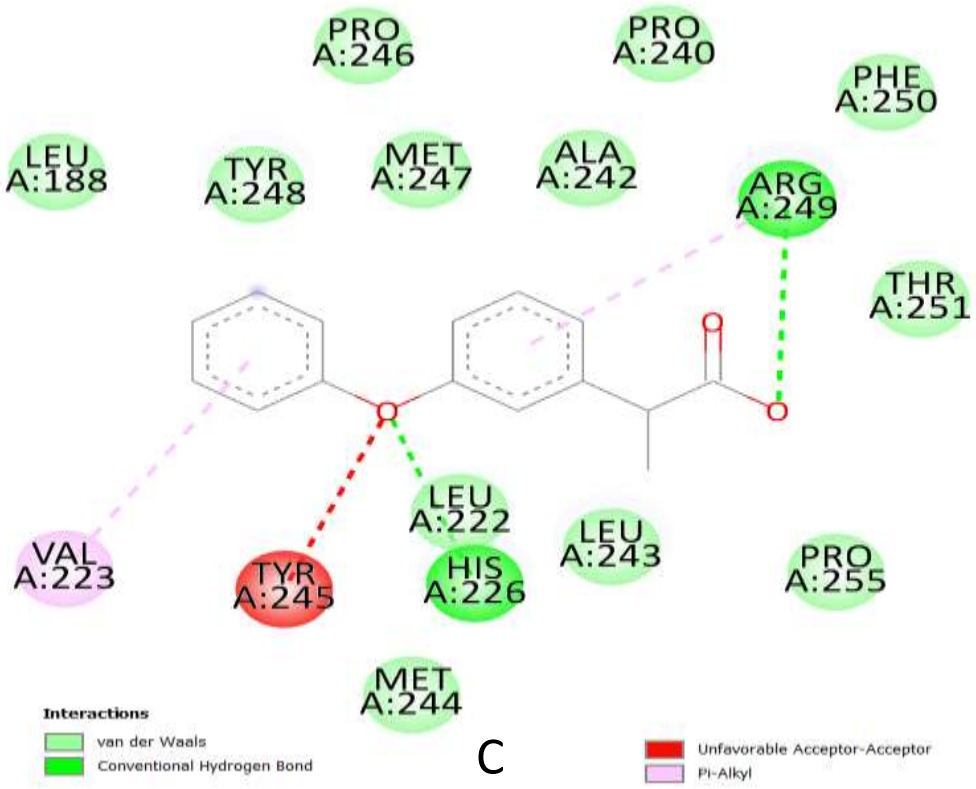
Halogen (Fluorine)
Unfavorable Donor-Donor
Pi-Pi Stacked
Pi-Pi T-shaped
Pi-Alkyl

B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LEU A:243
VAL A:223
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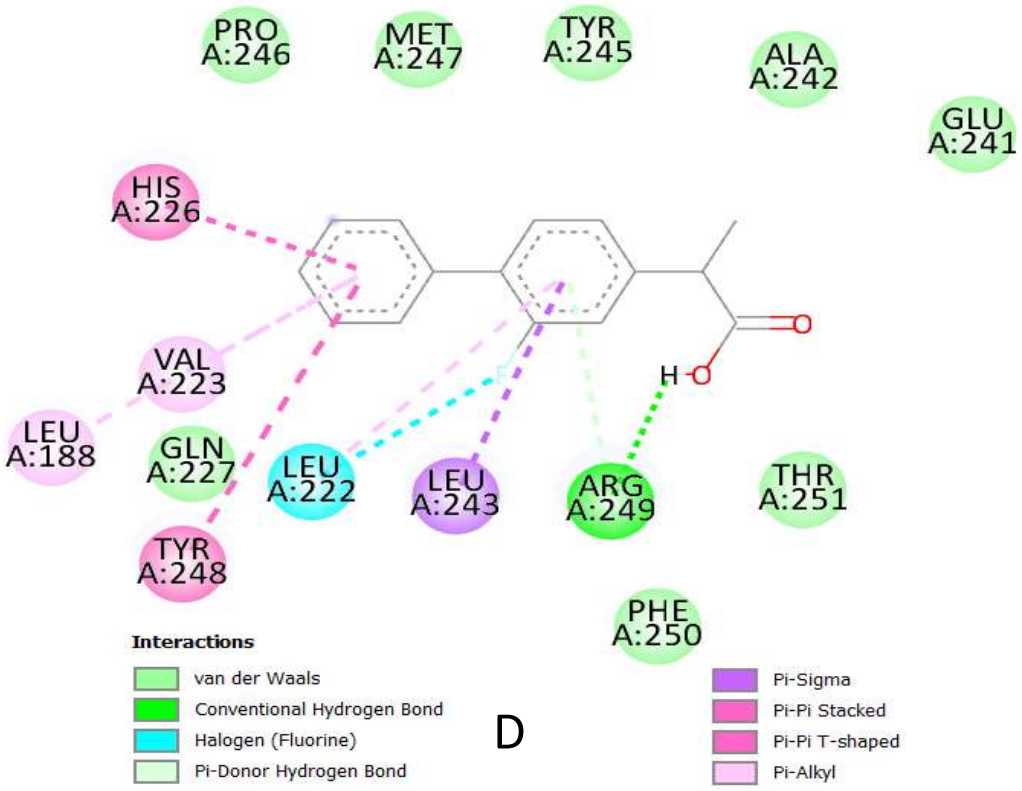
MET A:244
Interactions
van der Waals
Conventional Hydrogen Bond
Unfavorable Acceptor-Acceptor
Pi-Alkyl

C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Interactions
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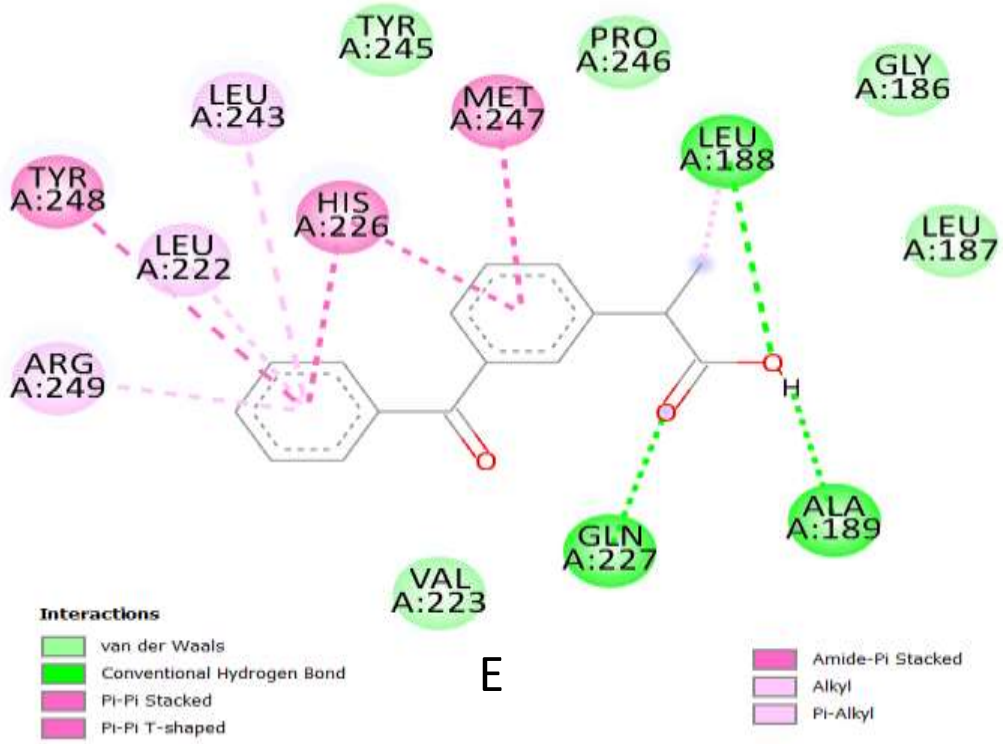
Halogen (Fluorine)
Pi-Donor Hydrogen Bond
Pi-Sigma
Pi-Pi Stacked
Pi-Pi T-shaped
Pi-Alkyl

D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ARG A:249
ALA A:189
GLN A:227
VAL A:223
Interactions
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Conventional Hydrogen Bond
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Pi-Pi T-shaped
Amide-Pi Stacked
Alkyl
Pi-Alkyl

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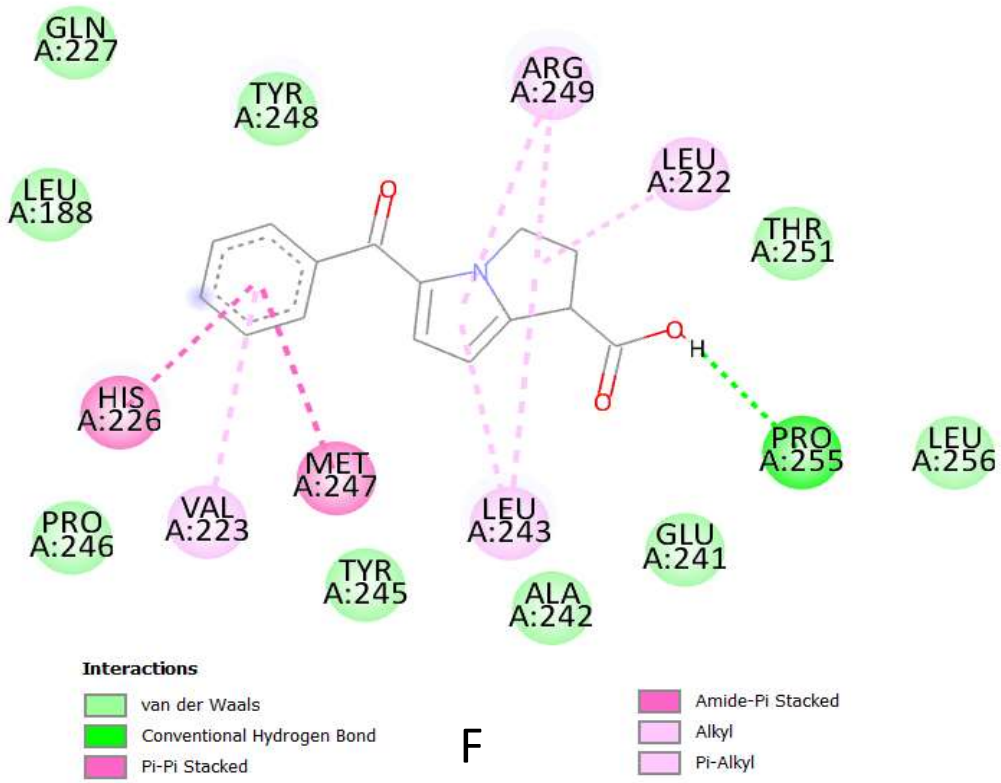
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VAL A:223
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GLU A:241
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Interactions
van der Waals
Conventional Hydrogen Bond
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Amide-Pi Stacked
Alkyl
Pi-Alkyl

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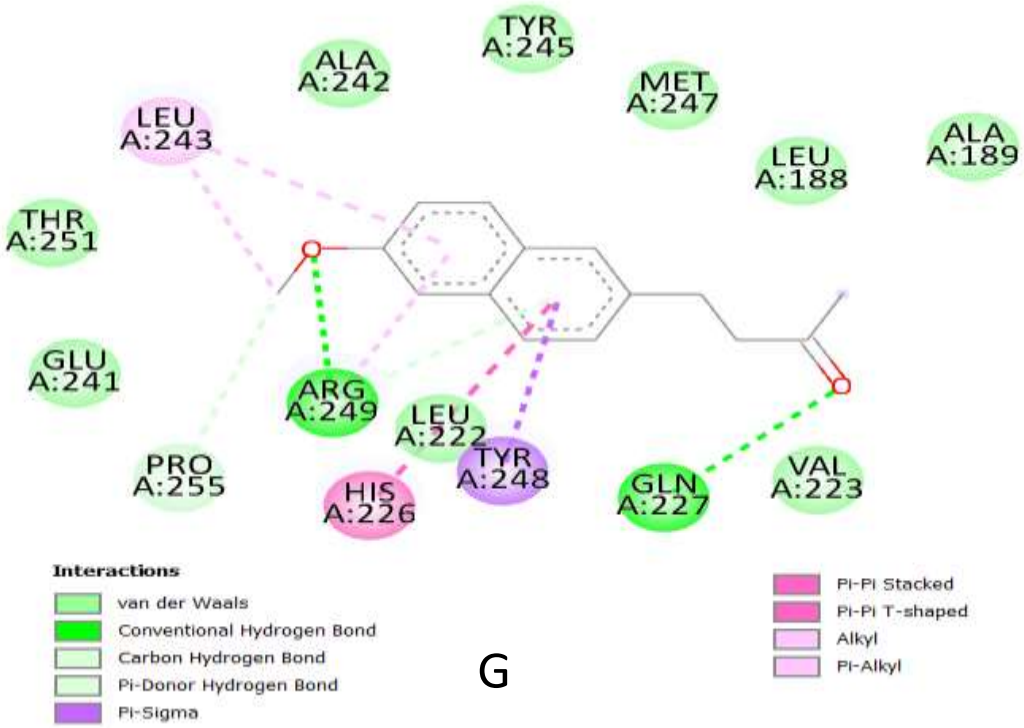
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PRO A:255
HIS A:226
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VAL A:223
Interactions
van der Waals
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Pi-Alkyl

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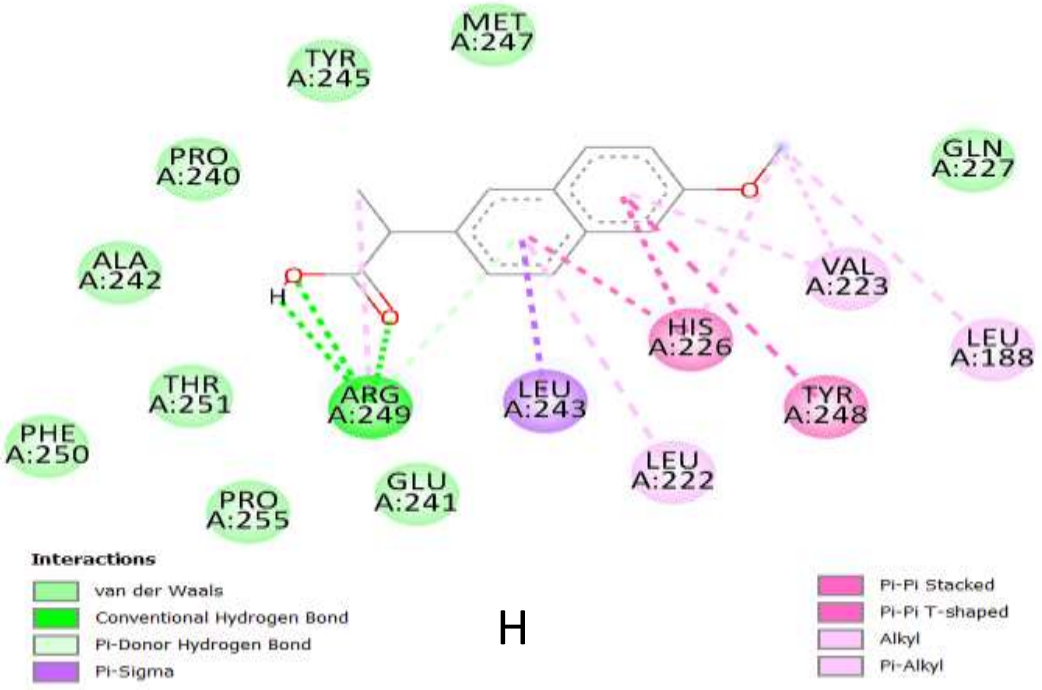
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PHE A:250
LEU A:222
GLU A:241
PRO A:255
Interactions
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Pi-Sigma
Pi-Pi Stacked
Pi-Pi T-shaped
Alkyl
Pi-Alkyl

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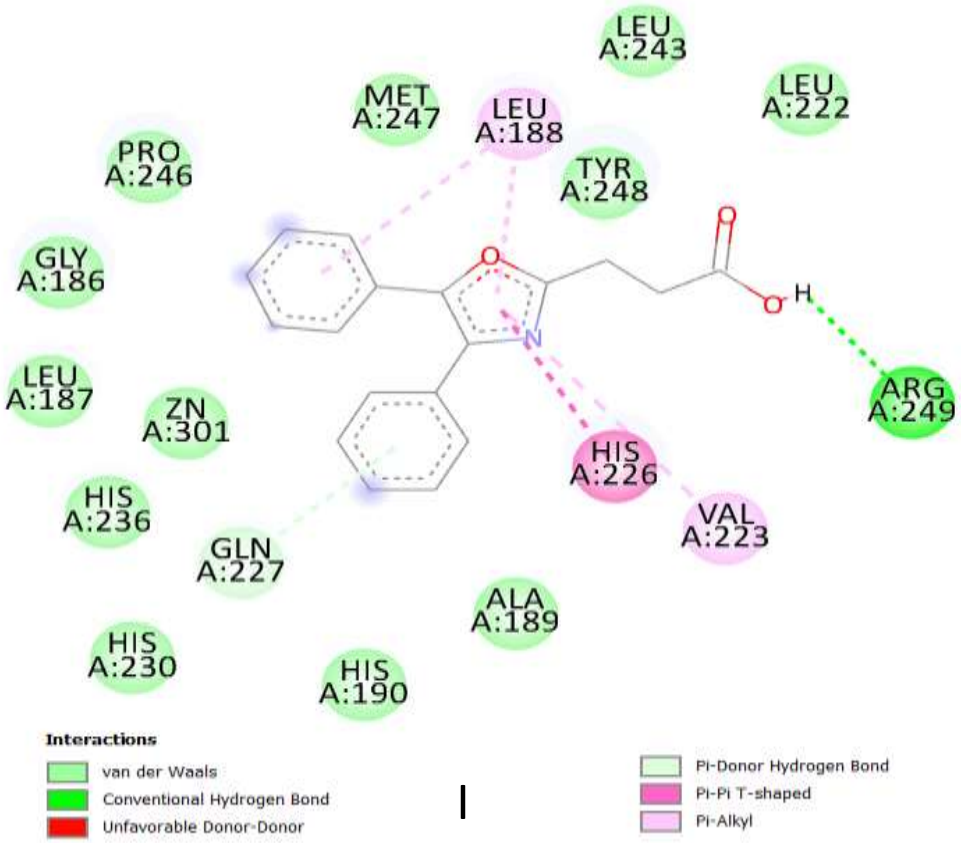
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VAL A:223
GLN A:227
ALA A:189
HIS A:230
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Interactions
van der Waals
Conventional Hydrogen Bond
Unfavorable Donor-Donor
Pi-Donor Hydrogen Bond
Pi-Pi T-shaped
Pi-Alkyl

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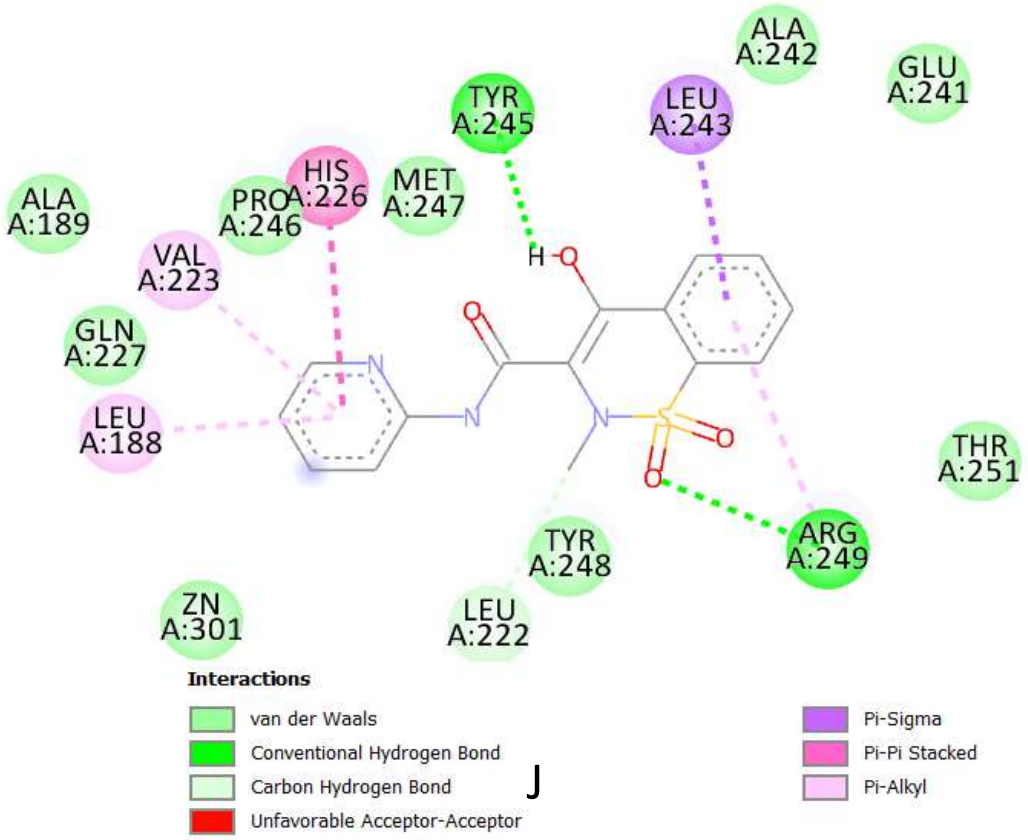


Fig. 12: - 2-D Molecular Docking View of NSAID with MMP-9 (6ESM) - (A) Celecoxib, (B) Diflunisal, (C) Fenoprofen, (D) Flurbiprofen (E) Ketoprofen (F) Ketorolac, (G) Nabumetone, (H) Naproxen, (I) Oxaprozin (J) Piroxicam.

Table 10: - Amino acids interaction, Hydrogen bond formation, and Binding energies of MMP-9 (6ESM) with 10 Selected NSAIDs.

S. No.	Ligands	Amino acids interaction	Amino acid with hydrogen bond interaction	Hydrogen bond length	Binding energies (kcal/mol)	Ki (µM)
1	CELECOXIB	GLY186, PRO246, VAL223, TYR248, LEU243, ARG249, MET247, LEU222, TYR245, HIS226, HIS236, HIS230, ALA189, ALA191, GLN227, HIS190, LEU187, LEU188	**ARG249, MET247, GLN227**	2.91, 2.96, 3.03	-12.42	0.0008

2	DIFLUNISAL	LEU222, LEU243, GLN227, LEU188, HIS226, VAL223, TYR248, MET247, PRO246, TYR245, ALA242, PRO240, GLU241, ARG249, PRO255, PHE250, THR251	**ARG249**	3.03	-12.42	0.0008
3	FENOPROFEN	PRO240, ALA242, MET247, PRO246, TYR248, LEU188, VAL223, TYR245, MET244, HIS226, LEU222, LEU243, PRO255, THR251, ARG249, PHE250	**ARG249, HIS226**	2.94, 2.56	-11.31	0.0051
4	FLURBIPROFEN	GLU241, ALA242, TYR245,MET247, PRO246, HIS226, VAL223, LEU188, GLN227, TYR248, LEU222, LEU243, ARG249, PHE250, THR251	**ARG249**	2.77	-12.56	0.0006
5	KETOPROFEN	PRO246, MET247, TYR245, HIS226, LEU243, LEU222, TYR248, ARG249, VAL223, GLN227, ALA189, LEU187, LEU188, GLY186	**LEU188, GLN227, ALA189**	2.75, 3.11, 2.92, 3.05	-12.56	0.0006
6	KETOROLAC	ARG249, TYR248, GLN227, LEU188, HIS226, PRO246, VAL223, MET247, TYR245, LEU243, ALA242, GLU241, PRO255, LEU256, THR251, LEU222	**PRO255**	3.04	-12.12	0.0013

7	NABUMETONE	TYR245, ALA242, LEU243, THR251, GLU241, PRO255, ARG249, LEU222, HIS226, TYR248, GLN227, VAL223, ALA189, LEU188, MET247	**ARG249, GLN227**	2.89, 3.18, 3.18	-11.54	0.0035
8	NAPROXEN	MET247, TYR245, PRO240, ALA242, THR251, PHE250, PRO255, GLU241, ARG249, LEU243, LEU222, HIS226, TYR248, LEU188, VAL223, GLN227	**ARG249**	2.94	-11.6	0.0031
9	OXAPROZIN	LEU222, LEU243, TYR248, LEU188, MET247, PRO246, GLY186, LEU187, HIS236, GLN227, HIS230, HIS190, ALA189, HIS226, VAL223, ARG249	**ARG249**	2.74	-12.98	0.0003
10	PIROXICAM	GLU241, ALA242, LEU243, TYR245, MET247, HIS226, PRO246, VAL223, ALA189, GLN227, LEU188, LEU222, TYR248, ARG249, THR251	**ARG249, TYR245**	2.64, 2.44	-12.98	0.0003

4.3.3 Molecular docking of combination of NSAIDs

The synergistic effect of these compounds for inhibition of MMP-9 was observed by combination docking of the selected compounds into the active site of the MMP-9. The two compounds that exhibited high negative binding affinities in individual

molecular docking were considered for evaluation of the synergistic inhibition effect. The binding energy of the combination docking was the same as that of the dockings performed individually. The Oxaprozin-Piroxicam combination gave the binding energy of -12.98 kcal/mol with MMP-9 showing consistency of interactions of the two compounds (Table 11 and Fig. 13 - 14). The combination of ligands formed 2 hydrogen bonds of which one was with Ala191 and the other with His226 which participates in the coordination complex with the Zn ion at the active site. Thus, our analysis of NSAIDs suggested that the identified combination of NSAIDs can convey highly effective synergistic structural inhibition of MMP-9 by binding at the active site of the enzyme.

Table 11: Binding energies of (MMP-9) with Oxaprozin – Piroxicam combination.

NSAIDs Combination and 6ESM	Highest Binding Energy (Kcal/mol)
Oxaprozin-Piroxicam	-12.98 kcal/mol

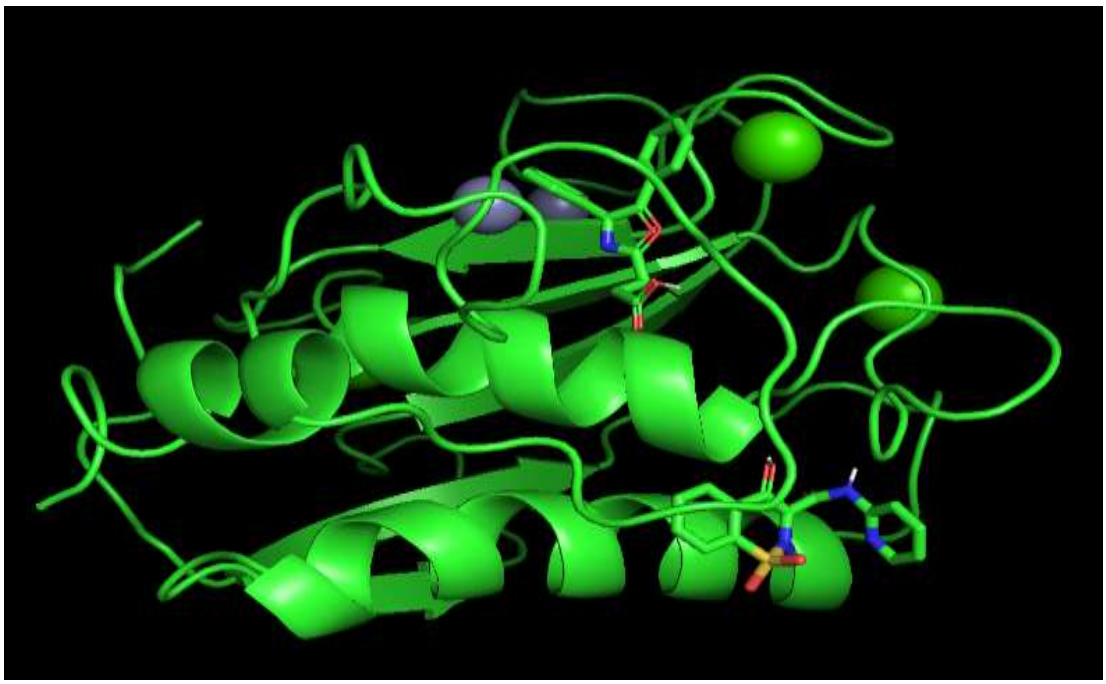


Fig.13: - Docking conformation of MMP-9 (6ESM) with Oxaprozin – Piroxicam Combination.

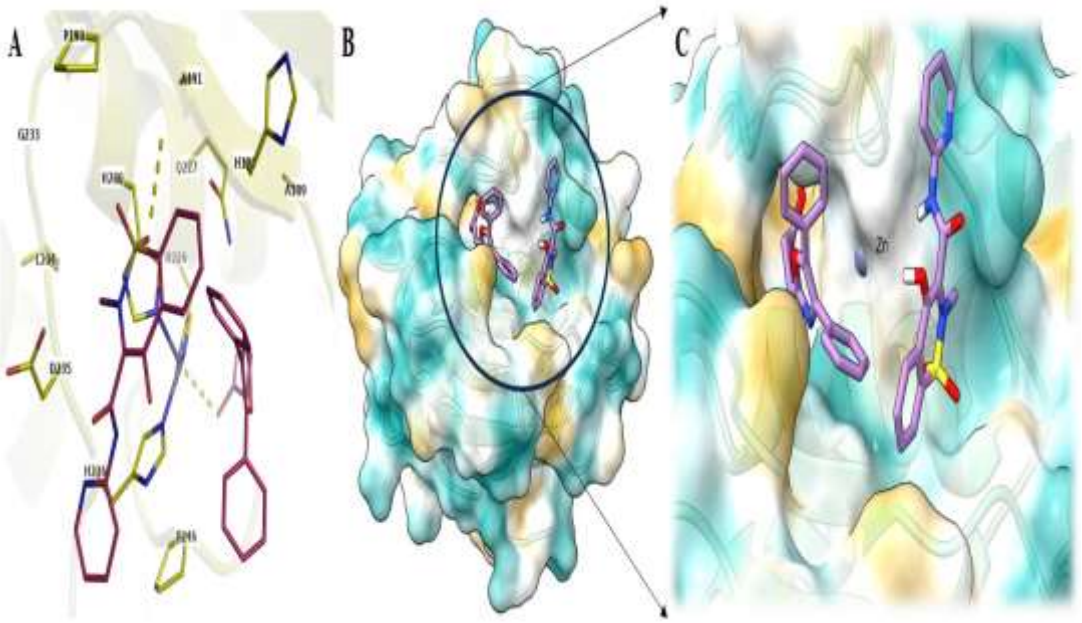


Fig. 14: - Molecular Docking interaction of MMP-9 (6ESM) with Oxaprozin – Piroxicam Combination.

4.4 Conclusion

The present study evaluated the physiochemical and biological properties of NSAIDs by PCA analysis. The docking binding energies in the present study were highly significant compared to previous studies and thus showed the immense potential of the selected NSAIDs to inhibit MMP-9. The combination analysis of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) for inhibiting MMP-9 revealed that the combination of Oxaprozin-Piroxicam with MMP-9 was the most effective one. Oxaprozin and Piroxicam revealed high affinity in both individual and combination dockings and the docking conformations were consistent blocking the active site of the MMP-9 thus inhibiting its activity.

CHAPTER – 5

Molecular Docking and In Vitro Evaluation of Luteolin and Piroxicam Reveal Synergistic Anticancer Potential

5.1 Introduction

Cancer is a highly complicated disease that influences a large number of people and is a prime cause of death in the world (9.7 million cancer deaths in 2022), with about 78 % of cases diagnosed in individuals aged 55 and older. The most common types of fatal cancers vary between men and women, with lung, stomach, liver, colon, and breast cancer being the most frequent. Worldwide, cancer deaths are projected to rise, with an estimated 12 million deaths expected annually by 2030 (Sainz et al., 2012).

Free radicals are normally reactive oxygen species (ROS) and reactive nitrogen species (RNS), which oxidize cellular proteins, nucleic acids, and lipids. Lipid peroxidation is a process where free radicals cause damage to polyunsaturated fatty acids. This process involves the propagation of oxidative damage. It can be terminated by enzymes such as glutathione reductase, glutathione peroxidase, and superoxide dismutase (Schattler et al., 1998) or antioxidants present in the body that scavenge free radicals. (Cheeseman & Slater, 1993). While the body has antioxidant defences to manage these free radicals, an excess can lead to oxidative and nitrosative stress. This chronic stress is linked to several diseases, including cancer, highlighting the importance of maintaining a balance in the body's redox system. It is investigated that ROS may cause the breaking of the DNA strand, and oxidative damage to the nucleotides, causing mutagenesis, resulting in cancer. Cancer cells have high levels of reactive oxygen species (ROS), that may cause DNA damage and cell death. Increased levels of ROS cause oxidative stress, damaging proteins, lipids, DNA and mitochondria (Pizzino et al., 2017), with DNA being particularly vulnerable. This damage can lead to genomic instability and cancer progression, Recent studies on treatment called NCX4040 (a nitric oxide donor) generates ROS, may destroy tumor cells (Sinha et al., 2022). Thus, Oxidative stress and inflammation are related to cancer and apoptosis tumor cells (Reuter et al., 2010).

A moderate accumulation of ROS can support tumor growth, (Moloney & Cotter, 2018), while excessive ROS or insufficient clearance leads to oxidative stress, (Perillo

et al., 2020), causing damage to DNA, which can promote cancer. Guanine is particularly vulnerable to oxidation, resulting in products like 8-oxoguanine that linked to tumorigenesis (Burrows & Muller, 1998), (C. Li et al., 2022). The base excision repair pathway is crucial for repairing oxidative DNA damage, if it fails, the likehood of mutation rises can cause tumor induction (Boiteux et al., 2017). additionally, cancer cells can adapt to higher ROS levels by enhancing their antioxidant defences, which further support cancer progression. Thus, a moderate increase in ROS is seen as beneficial for cancer transformation.

Excessive generation of reactive oxygen derivatives are linked to cancer development and progression (Circu & Aw, 2010), (Feng et al., 2020). High oxygen radicals levels are associated with various malignancies. Factors such as adaptation to low oxygen, metabolic changes, oncogenic mutation, and activation of pro-tumor signaling contribute to tumor formation. Hypoxia induced ROS control the expression of MMP-2 and MMP-9. It also promotes proliferation, migration and invasion of glioblastoma. Thus, it has been specially noted as a significant factor in this process.

Excessive concentration of free oxygen species may lead to cell-cycle arrest and apoptosis. To counteract this, cancer cells activate the transcription of antioxidants enzymes (Perillo et al., 2020). The nuclear erythroid 2-related factor (NRF2) act as a key factor in regulating antioxidants response (Sporn & Liby, 2012). NRF2 is often overexpressed in cancer, promoting cell survival by regulating the antioxidant system. Normally NRF2 is degraded by KEAP1, but under oxidative stress, it separates from KEAP1, moves to the nucleus, and activates antioxidant response elements (ARE) in target genes (Kansanen et al., 2013). These genes include those for various antioxidant enzymes, such as NAD(P)H Quinone dehydrogenase 1 and catalyse (Ma, 2013). Thus, cancer cells prevent themselves from excessive ROS.

Reactive oxygen species (ROS) may perform oxidative DNA damage, leading to double-stranded breaks and the creation of mutagenic 8-oxo-7-hydroxy-2-deoxyguanosine (8-oxodG). This compound is a significant contributor to spontaneous mutagenesis, as it can cause the conversion of guanine to thymine by pairing with cytosine and adenine (Sallmyr et al., 2008), (Oka & Nakabeppu, 2011). The build-up of 8-oxodG in cellular genome is a factor for cancer development.

Iron is a root source of ROS production and perform a key function in cell death across various organism and pathological conditions (Dixon & Stockwell, 2013). it is considered as a contributor component in the development of several cancers due to iron-induced oxidative stress (Toyokuni, 2016). The clinical impact of excess iron-induced ROS in cancers, emphasizing the connection between iron-induced ROS and carcinogenesis. The reducing therapeutic iron levels and lowering ROS can improve liver health and decreases HCC risk in liver cancer patients (Kato et al., 2007).Antioxidants help mitigate this damage by breaking the chains formed by these free radicals either by donating a hydrogen atom or an electron. Many of the investigations suggested that vegetables, fruits, and plants contain natural substances such as flavonoids, which have an antioxidant effect and can reduce the potential stress generated by reactive oxygen species. Approximately 4000 flavonoids have been found to date. (AQIL et al., 2006) The protective role of flavonoids in biological systems is attributed to their capacity to donate electrons to free radicals, bind metal catalysts, stimulate antioxidant enzymes, and neutralize alpha-tocopherol radicals, and inhibit oxidases. The common flavonoids included in DPPH and MTT assay study were Luteolin, Apigenin, and Quercetin. They have significant health benefits in various studies, such as luteolin has potential use as a chemopreventive agent against chromium-induced cancer by scavenging ROS and modulating cell signalling in human bronchial epithelial cells (Pratheeshkumar et al., 2014). It may also have medicinal benefits for cognitive dysfunction in Alzheimer's disease (Fu et al., 2014), and can positively influence liver carcinogenesis by reducing mast cell recruitment (Balamurugan & Karthikeyan, 2012). Apigenin have antioxidative properties and chelating redox-active metals. Apigenin's antioxidative activities are linked to its ability to donate hydrogen ions and electrons, which helps to stop the production of free radicals and prevent oxidative damage by scavenging free radical (Abdulla et al., 2017). The antioxidant mechanism of apigenin, highlighting its ability to enhance bioavailability and inhibit oxidative enzymes. The major in-vitro methods for assessing Apigenin's antioxidant potential include DPPH, ORAC and ABTS. (Kashyap et al., 2022). There is limited information available on Apigenin's antioxidant properties and discussion on its effects and mechanisms of action. Quercetin has been researched for its biological activities, including antioxidants, anti-inflammatory, antitumor (Y. Li et al., 2016). Quercetin can impede growth of cancer

cell by causing cell cycle arrests at G2/M or G1 phase and promoting the activity of enzymes and ROS in cells (Seufi et al., 2009). It activates ROS-scavenging enzymes for the reduction of intracellular ROS level (N. Li et al., 2014). Pure Quercetin have higher antioxidant activity. Due to the contribution of hydroxyl groups. The radical inhibitory and metal reducing activity of quercetin decreases when cations are chelated. It utilized three methods including DPPH. The metal ions significantly alter the chemical properties, affecting its antioxidant activity. Quercetin may reduce Fe (III) in a concentration and time dependent manner (Dolatabadi et al., 2014).

Various assays are employed to assess the antioxidant activity of herbal extracts and phenolic compounds, utilizing different radicals and methods to analyze antioxidant effects and determine oxidation products. The most potent method involves using a stable free radical, DPPH, to assess how well antioxidants can neutralize reactive species. The ability of antioxidants to reduce DPPH is a key feature of this method, as a single electron of the nitrogen atom in DPPH is reduced by hydrazine by taking a hydrogen atom from the antioxidants. The DPPH radical is intensely coloured and stable; due to this property, its solution is commonly used. It is identified that the UV-vis spectrum of DPPH shows two distinct bands due to π-π^* transitions with the unpaired electron contributing significantly to the visible band (O. Chen et al., 2009). When DPPH is mixed with a hydrogen atom donor substance solution, its violet colour fades, indicating the formation of the reduced DPPH radical (DPPH-H) (Yapıcı et al., 2021). This colour change from violet to pale yellow occurs due to radical reduction by antioxidants, examined by using UV-vis spectroscopy and to evaluate the antioxidant property of substances like herbal extracts and phenolic compounds (Xie & Schaich, 2014).

The DPPH test is used to estimate the total content of reductants in plant extracts, indicating the antioxidant capabilities of phenolic compounds and their capacity is quantified (Gulcin, 2020), (Gülçin, 2011).

$$XO^* + ROOH \longrightarrow XOH + ROO$$

This method is known for being simple, sensitive, fast, and reproducible, making it a convenient choice for evaluating the antioxidant potential of various compounds and herbal extracts. The concentration referred to as $IC_{50,}$ indicates its efficiency or

inhibitory capacity. The IC_{50} values are essential for comparing the radical scavenging capacities of various antioxidants.

The MTT assay, developed in 1983, is widely utilized to determines viability of cells and metabolic activity (Mosmann, 1983).The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) consists of a positively charged tetrazole ring surrounded by aromatic rings. When reduced by metabolically active cells, MTT is converted into a violet-blue insoluble molecule called formazan (Berridge et al., 2005), (Stockert et al., 2018). This reaction allows for colorimetric measurement of cell metabolic activity. While mitochondria are often associated with MTT reduction, (Surin et al., 2017), (Stockert et al., 2018). Various studies have found formazan in multiple cellular organelles, including the endoplasmic reticulum, lipid droplets, plasma membranes, nucleus, and microsomes (Stockert et al., 2012) (Bernas & Dobrucki, 2000), (Y. Liu et al., 1997). In an MTT assay, the IC50 value represents the concentration of a drug or compound needed to inhibit a biological process by 50% and indicates the potency of the drug.

The present study deals with in vitro investigation of natural phytochemicals for their antioxidant and, Anticancer activity toward cancer cell lines. Further analysis in in-vivo conditions can provide safe, natural and effective treatment against cancers.

5.2 Methodology

5.2.1 Molecular Docking analysis of MMP-9

Molecular docking studies were performed to evaluate the binding interactions of selected flavonoids (Quercetin, Luteolin, and Genistein) and NSAIDs (Ketorol and Piroxicam), both individually and in combination, against matrix metalloproteinase-9 (MMP-9) based on our previous studies (Singh et al., 2024). The three-dimensional crystal structure of MMP-9 was retrieved from the Protein Data Bank (PDB). The ligands were obtained from the PubChem database and converted into PDBQT files. For docking, a grid box was constructed to cover the MMP-9 active sites, with dimensions large enough to accommodate ligand flexibility and ensure comprehensive exploration of the binding pocket. The Lamarckian Genetic Algorithm (LGA) was employed as the search method, with a population size of 150, maximum number of evaluations set to 2.5 × 10^6, and 100 independent docking runs for each ligand.

Docking results were ranked based on binding free energy (ΔG, kcal/mol). The most stable complexes were selected for further analysis. Protein–ligand interactions were visualized using PyMol. Comparative docking of combinations of flavonoids and NSAIDs was performed to assess potential synergistic binding interactions within the active site of MMP-9.

5.2.2 DPPH assay

Free radicals are molecules that can damage DNA, and contributing to aging and diseases like cancer and inflammation. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical is commonly used to test antioxidant activity because it changes colour from purple in methanol to yellow when it reacts with antioxidants, indicating the reduction process. The DPPH purple colour in methanol has maximum absorption at 517 nm, that decreases in yellow colour when it reacts with hydrogen to produce the reduced DPPH-H species. The produced electrons consequent decolorization are stoichiometric.

To measure flavonoids anticancer activity (Luteolin, Genistein, and Quercetin) and NSAIDs (Ketorol and Piroxicam) using the DPPH radical scavenging test. A small amount (0.5 mg/mL) of flavonoids and NSAIDs solution was mixed with 10 % (v/v) ethanol to obtain 100 µL were mixed to a test tube using a micro syringe and 1ml DPPH solution (100 µM) in 99.8% (*v*/*v*) ethanol and 1 mL of 96% (*v*/*v*) ethanol, then vortexed and incubation time for 30 minutes. The change in colour was measured at 517 nm to determine how well flavonoids and NSAIDs can neutralize free radicals. It is tested with gallic acid (0.05 mg/mL) and Trolox (1 mg/mL) for comparison. The percentage of DPPH radical inhibition was calculated by following expression to assess antioxidant effectiveness.

$$\text{Antioxidant Activity (\%)} = [\text{Abs Control} - \text{Abs Sample} / \text{Abs Control}] \times 100$$

The final results are shown as IC 50 values, which indicate the concentration of antioxidant or radical-scavenging agent needed to reduce the initial radical amount by 50 %. Linear regression analysis was used to determine these values from the concentration versus activity graphs. The spectrophotometric tests were performed in triplicate on both the samples and reference substances.

5.2.3 MTT assay

Many flavonoids can inhibit cancer cell growth. The MTT assay was used to monitor cell development and changes, showing by the flavonoids and NSAIDs, which was prominent in phytochemical and antioxidant tests. This experiment evaluates the anticancer potential of Flavonoids (Luteolin, Genistein, and Quercetin) with MCF-7 a human breast cancer cell line. The results compared to the NSAIDs (Ketorol, and Piroxicam), indicating that increasing flavonoids concentrations increases cell death, proposing as an anticancer agent.

The testing of the cytotoxic effects of flavonoids and NSAIDs on breast cancer cells (MFC-7). The samples were dissolved in DMSO and applied to cells cultured in 96-well plates. After 24 hours, the medium was replaced, and cells were incubated for an additional 24 or 48 hours with different concentrations of the samples. The MTT assay, which involves adding MTT solution (5 mg/mL), incubating 3 hours, and then processing the plates further with 10 % SDS buffer (100 µL) were mixed in each well, incubate overnight then absorbance was determined at 570 nm with the help of microplate reader. The current study determines the potential substances which kill cancer cells.

5.2.4 ROS Assay

Intracellular ROS levels were quantified using the Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, UK) with the fluorogenic dye H2DCFDA, following the manufacturer's protocol. Breast cancer cells (25,000/well) were inoculated in 96-well black-wall plates (Corning, USA) and incubated overnight. On the subsequent day, cells were washed with HBSS (150 µl; Gibco, UK) and incubated with staining buffer (100 µl; 20 µM H2DCFDA in HBSS) for 40 min at 37 °C. After washing, HBSS (100 µl) was added, and fluorescence was measured using a POLARstar Omega reader at 485 nm excitation and 535 nm emission. For treatment-induced ROS measurement, compounds (flavonoids and NSAIDs) were added along with HBSS, and fluorescence was recorded after the desired incubation time.

The viability of treated MCF-7 cells was expressed in percentage of control cell viability. Each test was repeated three times, and results are shown as mean ± SD. Data analysis was performed using GraphPad Prism software.

5.3 Results and Discussion

5.3.1 Molecular docking of flavonoids and NSAIDs

Molecular docking of the Quercetin, Luteolin, Genistein, Ketorol and Piroxicam was performed individually as well as in combination of one flavonoid and one NSAID. These flavonoids and NSAIDs were selected based on our previous analysis conducted separately for inhibition of MPP-9 (Singh et al., 2024). the docking of piroxicam-luteolin combination gave the highest negative binding affinity of -6.89 kcal/mol (Table 12), indicating the effective inhibition of MMP-9. To further explore the inhibition potential and to evaluate the antioxidant effect of the best flavonoids and NSAIDs *in vitro,* DPPH assay, MTT assay, and ROS assay were performed both individually and in combinations.

Table 12: Amino acids interaction, Hydrogen bond formation, and Binding energies of MMP9-flavonoid-NSAID complex

S. No.	Combination of NSAID and Flavonoid	Amino Acids Interaction	Binding Energy (Kcal/mol)
1	Piroxicam-Luteolin	GLU241, ALA242, LEU243, TYR245, MET247, PRO245, HIS226, GLN227, ALA189, LEU188, LEU222, TYR248, ARG249	-6.89 kcal/mol

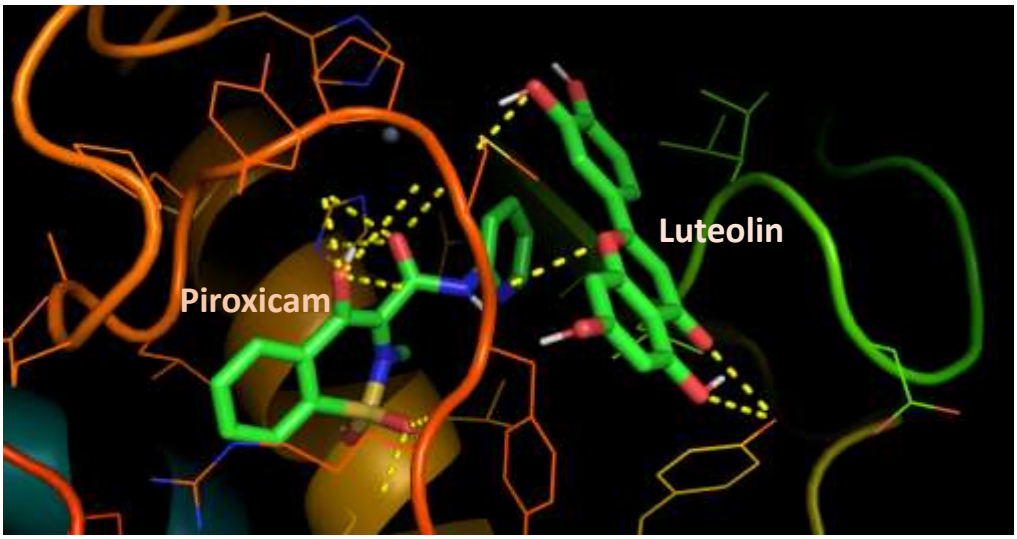


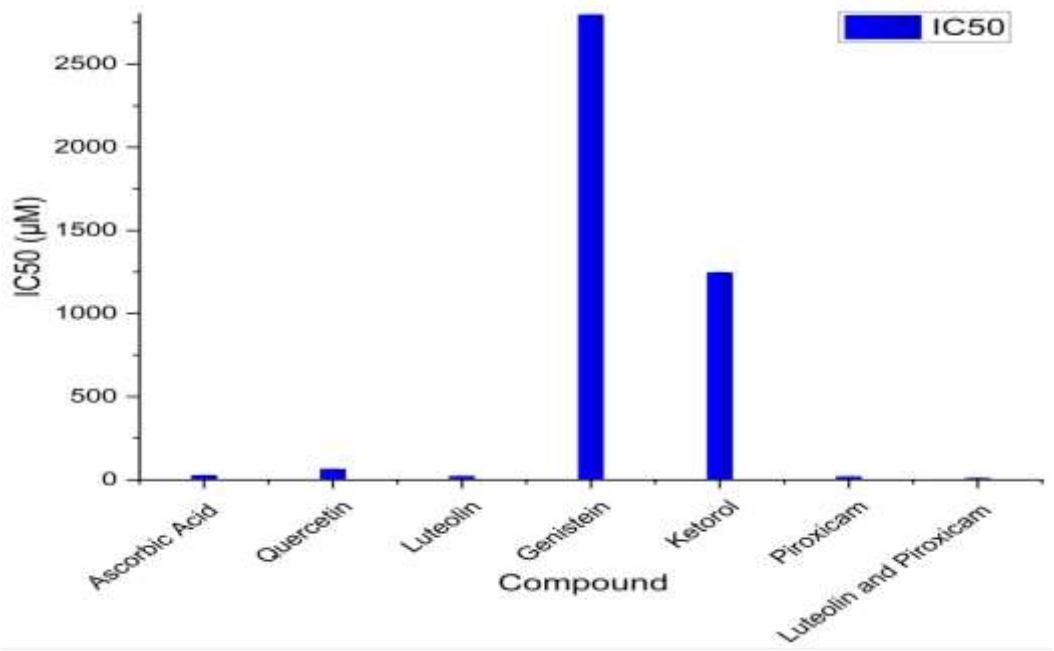
Fig 15: Molecular docking view of Piroxicam – Luteolin combination

5.3.2 DPPH Assay

The samples were analysed to determine their antioxidant potential, and the 50% inhibitory concentration (IC_{50}) values were calculated to identify the most potent flavonoids and non-steroidal anti-inflammatory drugs (NSAIDs) demonstrating effective inhibition of MMP-9. Among the compounds screened, the flavonoids and NSAIDs exhibiting the most favourable binding energies in combined docking studies (Table 13). In individual analyses, luteolin and piroxicam displayed highly significant antioxidant effects, with IC_{50} values of 22.85 ± 0.080 μM and 20.512 ± 0.04 μM, respectively. However, when tested in combination, luteolin and piroxicam produced a markedly reduced IC_{50} value of 10.89 ± 0.34 μM, indicating a substantially enhancement in antioxidant capacity as compared to their individual effects. The reduced IC_{50} value expressing the synergistic connection between luteolin, a naturally occurring flavonoid with well-documented antioxidant and anticancer properties, and piroxicam, an NSAID as an anti-inflammatory and potential anticancer effects. The observed synergy demonstrates that the combined administration of luteolin and piroxicam may significantly improve the mitigation of oxidative stress conditions that associated in cancer progression. Such results indicating the therapeutic potential of integrating natural compounds with conventional pharmacological agents to enhance overall efficacy, reduce required dosages, and potentially reduce side effects, thereby offering a strategy for developing novel combination therapies targeting oxidative mechanisms in cancer.

Table 13: Inhibitory concentration (IC50) values of best flavonoids and NSAIDs in DPPH assay

S. No.	Sample Name	Inhibitory Concentration (IC_{50}) Value (μM)
1	Ascorbic Acid	27.73 ± 0.018
2	Quercetin	65.46 ± 0.055
3	Luteolin	22.85 ± 0.080
4	Genistein	2798 ± 0.056
5	Ketorol	1248 ± 0.041
6	Piroxicam	20.512 ± 0.04
7	Luteolin and Piroxicam	10.89 ± 0.34



Graph 1: IC50 values of all considered compounds (Flavonoids and NSAIDs) in DPPH assay

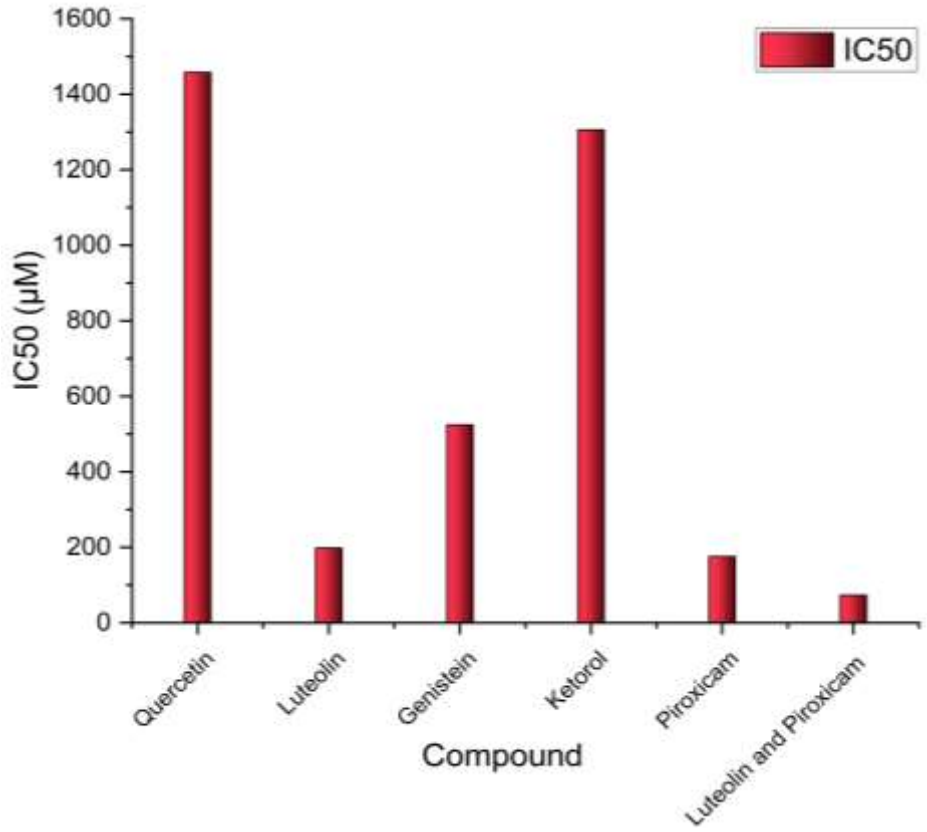
5.3.3 MTT Assay

The MTT assay is a widely used, sensitive, and reliable colorimetric technique to identify viability of cells, proliferation, and activation. It functions in metabolically active cells can convert the yellow, water-soluble compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble dark blue formazan crystals. The quantity of formazan produced is directly proportional to the number of viable cells, making this assay a robust quantitative measure of cytotoxicity. In this study, flavonoids and NSAIDs exhibiting the most favourable docked ligands were selected for evaluation against a human breast cancer cell line. All tested compounds demonstrated the ability to inhibit cancer cell proliferation to varying degrees. Notably, luteolin and piroxicam emerged as the most potent agents, showing individual IC_{50} values of 198.3 ± 0.088 µM and 175.5 ± 0.129 µM, respectively (Table 14). Further assessment of their combined effect revealed a remarkably reduced IC_{50} value of 73.3 ± 0.25 µM, indicating a pronounced synergistic cytotoxic effect. This substantial decrease in IC_{50} suggests that the luteolin–piroxicam combination significantly enhances the inhibition of cancer cell proliferation compared to either compound alone. The results highlight the potential of integrating natural flavonoids with conventional pharmacological agents to improve therapeutic outcomes, reduce

required doses, and potentially minimize toxicity. Such synergistic combinations could represent a promising approach for anticancer treatment strategies targeting cell proliferation mechanisms.

Table 14: **Inhibitory concentration (IC50) values of best flavonoids and NSAIDs in MTT assay**

S. No.	Sample Name	Inhibitory Concentration (IC_{50}) Value (µM)
1	Quercetin	1458 ± 0.107
2	Luteolin	198.3 ± 0.088
3	Genistein	524.5 ± 0.103
4	Ketorol	1306 ± 0.058
5	Piroxicam	175.5 ± 0.129
6	Luteolin and Piroxicam	73.3 ± 0.25



Graph 2: IC50 values of all considered compounds (Flavonoids and NSAIDs) in MTT assay

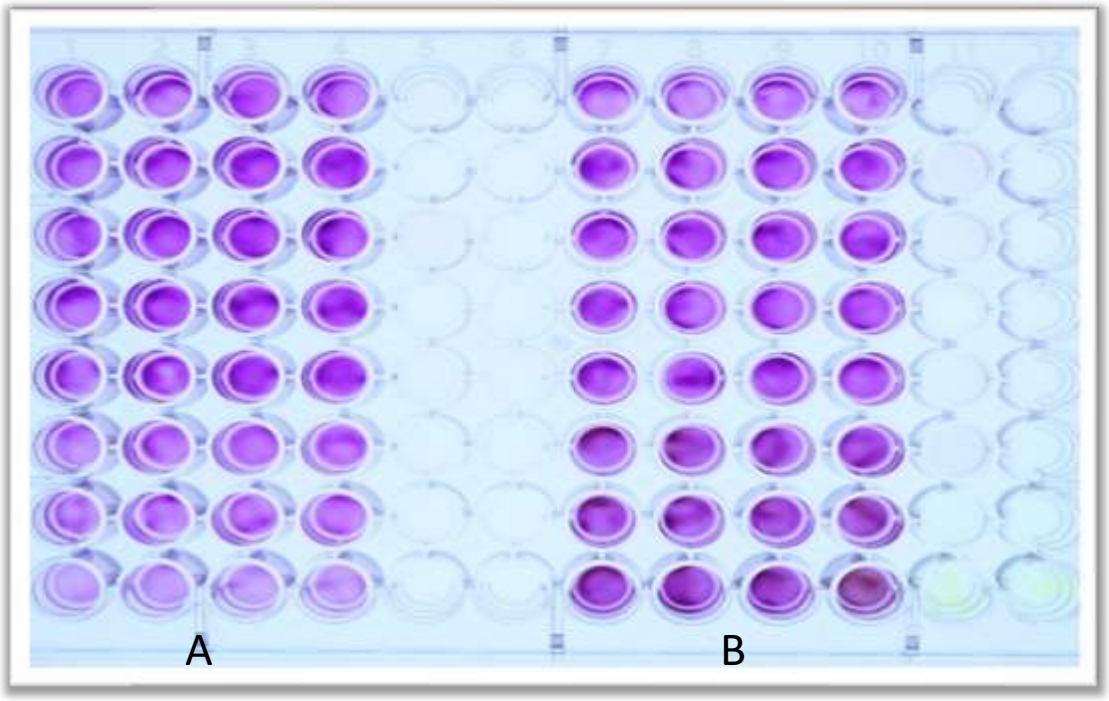


Fig 16: MTT Assay for (A) Luteolin (B) Piroxicam

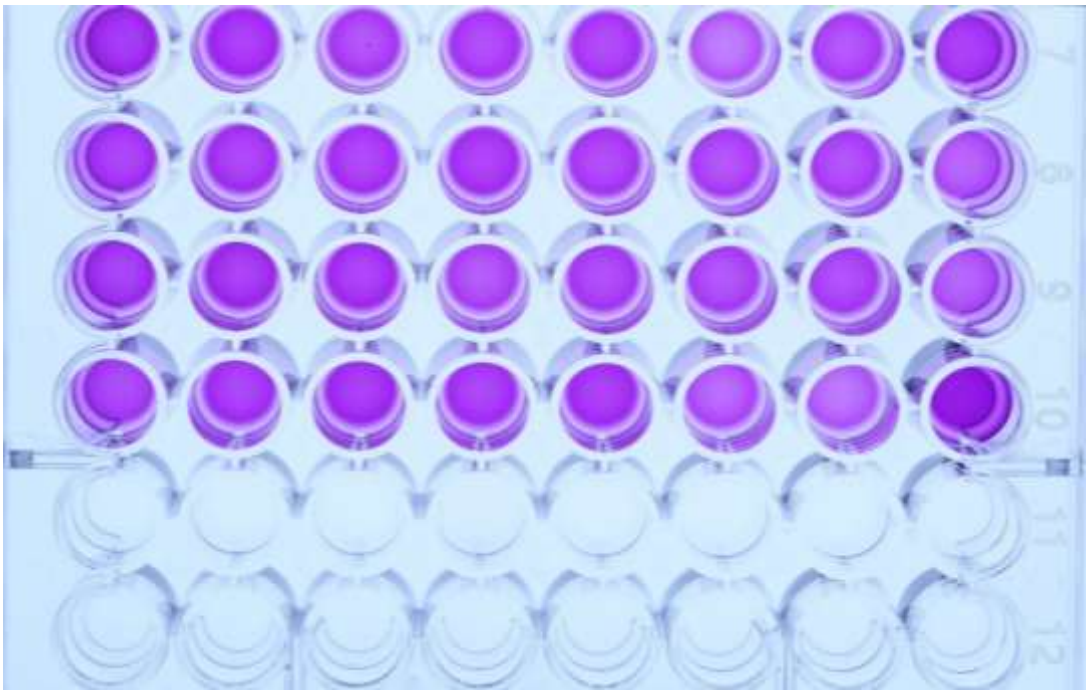


Fig 17: Luteolin-Piroxicam Combination MTT Assay

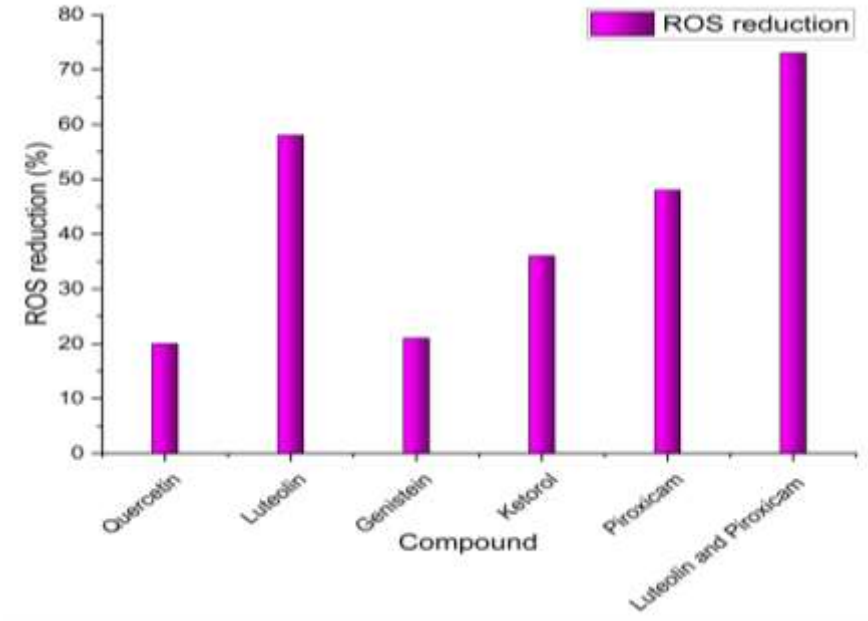
5.3.4 ROS Assay

The intracellular ROS levels were estimated in the absence and presence of best flavonoids and NSAIDs, and also in the presence of the best combination of luteolin and piroxicam. The percentage reduction in ROS levels compared to control were

evaluated based on the fluorescence recorded (Table 15). Effective reduction in ROS was observed in luteolin and piroxicam individually. This reduction was observed to be amplified on the combination of compounds.

Table 15: ROS reduction efficiency analysis of best flavonoids and NSAID

S. No.	Sample Name	ROS reduction (%)
1	Quercetin	20
2	Luteolin	58
3	Genistein	21
4	Ketorol	36
5	Piroxicam	48
6	Luteolin and Piroxicam	73



Graph 3: ROS reduction by considered compounds (Flavonoids and NSAIDs)

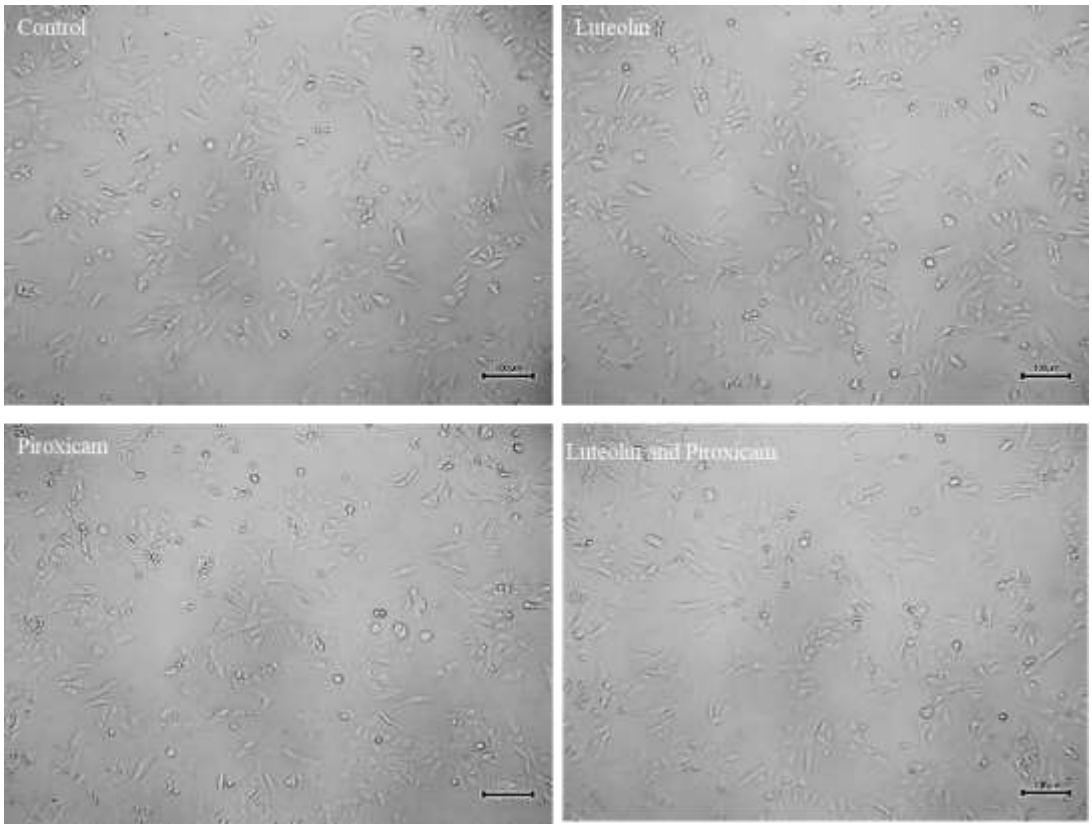


Fig 18: - Comparative evaluation of luteolin and piroxicam, treated individually and in combination, on MCF-7 cells, revealing enhanced reduction in cell population with the combination treatment in comparison with control.

5.4 Conclusion

The present investigation demonstrates that the combination of luteolin, a natural flavonoid, and piroxicam, a widely used NSAID, exerts synergistic antioxidant and cytotoxic effects against cancer cell lines. Molecular docking confirmed favorable binding interactions of the luteolin–piroxicam complex with key residues, supporting their strong binding affinity. In vitro assays studies further confirmed that while both compounds individually exhibited significant antioxidant and cytotoxic activities, their combination markedly reduced IC_{50} values, thereby enhancing their overall efficacy. The synergistic reduction in intracellular ROS levels further highlights their ability to modulate oxidative stress, a critical factor in cancer progression. Collectively, these results suggest that the luteolin–piroxicam combination holds considerable promise as a safe, natural, and effective anticancer strategy. However, as this study was limited to in vitro analysis, further in vivo validation and mechanistic studies are essential to fully establish its therapeutic potential and clinical applicability in cancer treatment.

CHAPTER – 6

CONCLUSION

Cancer remains one of the most devastating health challenges globally, with high morbidity and mortality rates driven by its aggressive proliferation and metastatic capabilities. Among molecular targets, matrix metalloproteinase-9 (MMP-9) has gained significant attention due to its central role in extracellular matrix degradation, angiogenesis, and tumor invasion. This study explored the inhibitory potential of natural flavonoids and nonsteroidal anti-inflammatory drugs (NSAIDs) against MMP-9, both individually and in combination. In-silico screening identified luteolin and quercetin as the most promising flavonoids, while oxaprozin and piroxicam emerged as the top NSAIDs. Molecular docking revealed that certain flavonoid–flavonoid and NSAID–NSAID combinations displayed stronger binding affinities than individual compounds, with cross-class pairing of luteolin and piroxicam also demonstrating high inhibitory potential. These computational results were complemented by experimental validation, where DPPH radical scavenging, ROS reduction and MTT assays confirmed strong antioxidant activity and cancer cell cytotoxicity. Collectively, the findings suggest that the flavonoids and NSAIDs combination could effectively target MMP-9 and reduce oxidative stress–mediated cancer progression.

CHAPTER – 7

SIGNIFICANCE OF INVESTIGATION

Cancer progression is a multifactorial process involving uncontrolled cell proliferation, evasion of apoptosis, angiogenesis, and metastasis. Among the numerous molecular mediators implicated in tumor aggressiveness, matrix metalloproteinase-9 (MMP-9) has emerged as a pivotal enzyme responsible for extracellular matrix (ECM) degradation, facilitating tumor invasion and metastasis. Overexpression of MMP-9 has been reported in diverse cancer forms, namely breast, colorectal, lung, and pancreatic and other cancers, and is aligned with decreased survival rates, high metastatic potential, and chemoresistance. The oxidative stress has been exhibiting to upregulate MMP-9 expression through activation of transcription factors such as NF-κB and AP-1, thereby linking redox imbalance to cancer progression. Therefore, MMP-9 is therapeutic target for cancer intervention.

Natural compounds, particularly flavonoids such as luteolin and quercetin, have drawn significant interest due to their pleiotropic anticancer effects, including antioxidant, anti-inflammatory, and anti-metastatic activities. These phytochemicals shown to suppress MMP-9 expression at both transcriptional and post-translational levels, inhibit cancer cell invasion, and modulate multiple oncogenic pathways with minimal toxicity to normal cells. Similarly, nonsteroidal anti-inflammatory drugs (NSAIDs), including piroxicam and oxaprozin, exert anticancer activity through cyclooxygenase (COX) inhibition, suppression of prostaglandin synthesis, and modulation of MMP expression. Notably, NSAIDs can downregulate MMP-9, reduce inflammation-driven tumor progression, and synergize with other chemopreventive agents.

Despite the individual benefits of flavonoids and NSAIDs, combination therapy targeting MMP-9 has been underexplored. Rationally designed drug combinations can enhance therapeutic efficacy, and minimizing toxicity. In the context of MMP-9 inhibition, combining antioxidant-rich natural molecules with anti-inflammatory NSAIDs offers the potential to address both oxidative stress–mediated and inflammation-mediated upregulation of MMP-9. Such a dual approach may achieve

superior inhibition of tumor invasion, angiogenesis, and metastasis compared to monotherapy.

This investigation is significant for several reasons. First, it integrates computational and experimental strategies to identify potent MMP-9 inhibitors from two distinct drug classes. Molecular docking provides a structural basis for understanding binding interactions. Second, it evaluates both individual and combination effects, highlighting synergistic interactions that may be more effective. Third, it incorporates antioxidant (DPPH assay), ROS reduction and cytotoxic (MTT assay) evaluations, linking MMP-9 inhibition to oxidative stress reduction and direct cancer cell growth suppression.

From a translational perspective, this study opens new doors for the development of multi-targeted, low-toxicity anticancer regimens. Since both flavonoids and many NSAIDs are already well-characterized for safety, their repurposing in combination therapies could significantly reduce the time and cost required for clinical implementation. Furthermore, the results provide a foundation for **in vivo** studies and clinical trials aimed at validating efficacy, determining optimal dosage ratios, and assessing pharmacokinetic compatibility.

In conclusion, the significance of this work lies in its innovative approach to tackling MMP-9–driven cancer progression by combining natural antioxidants with established anti-inflammatory drugs. This dual-targeted strategy not only addresses the multifaceted regulation of MMP-9 but also offers a promising pathway toward more effective, safer, and accessible anticancer therapies.

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