

Integrating Phytochemical Profiling with Pharmacological Evaluation of Himalayan Mango (*Mangifera sylvatica* Roxb.) Leaves: GC-MS/MS Insights into Antioxidant, Anti-inflammatory, and Analgesic Potentials

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Abstract

Objectives: *Mangifera sylvatica* Roxb. (Himalayan mango) is a wild fruit species available in Bangladesh. Its therapeutic properties have been scientifically less explored; thus, the current research aimed to assess the phytochemical profiling, acute toxicity, antioxidant, analgesic, and anti-inflammatory properties of the ethanolic extract derived from *Mangifera sylvatica* leaves (EMS).

Method: The acute toxicity was evaluated through the *in vivo* method on Swiss albino mice. The antioxidant capacity was assessed using the DPPH radical scavenging assay and reducing power assessment. *In vitro* anti-inflammatory effects were determined through a protein denaturation test. The analgesic activity of EMS was examined using acetic acid-induced writhing and formalin-induced paw-licking tests on Swiss albino mice, along with a histopathological test. GC-MS/MS analysis was employed to identify bioactive compounds in the extract. Additionally, *in silico* studies, including pass prediction, ADME/T analysis, and molecular docking of various secondary metabolites, were conducted using different online tools.

Results: At high doses, the extract did not exhibit any notable toxic effects in the acute toxicity. There were no significant changes in the histopathological parameters of vital organs when treated with the extract. The results showed that EMS exhibited notable antioxidant activity in the DPPH assay, with an IC_{50} value of 348.72 μ g/mL, and demonstrated a concentration-dependent increase in reducing power. The extract also displayed significant anti-inflammatory effects, with an IC_{50} value of 142.52 μ g/mL, comparable to the standard drug diclofenac in the protein denaturation assay. At doses of 100, 200, and 400 mg/kg, EMS significantly reduced acetic acid-induced writhing and formalin-induced paw licking in mice in a dose-dependent manner ($p < 0.01$). Molecular docking analysis revealed that the compounds in the extract had binding affinities ranging from -0.3 to -10.8 kcal/mol toward human target receptors, indicating potential pharmacological activity. ADME/T analysis also supports the drug-likeness characteristics of identified phytochemicals.

Conclusion: Overall, the EMS exhibited no significant toxic effects in vital organs, excellent pharmacological activities with antioxidant, anti-inflammatory, and analgesic properties, which demands further extensive research.

1 | Introduction

Since ancient times, plants have been utilized to address various health conditions due to their safety and effectiveness (S. Alam et al., 2025) (Mohammad, Md. Anisul, et al., 2025). Numerous bioactive chemicals included in foods derived from plants are advantageous for health in addition to providing basic nourishment (Saxena et al., 2013). Numerous naturally occurring chemical components found in medicinal plants contribute to their therapeutic qualities. Additionally, a growing reliance on the use of medicinal plants in developed nations has been connected to the extraction and production of different drugs and chemotherapeutics from these plants, as well as from traditionally used herbal medicines in rural areas (Sofowora, 1996). With their potent pharmacological effects on animal systems and organs, alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenic, tannins, resins, lactones, quinines, volatile oils, and other chemical constituents of medicinal plants—particularly the secondary metabolites—have the power to alleviate suffering, cure diseases, and mend wounds, cuts, and burns. The World Health Organization (WHO) estimates that 1.5 billion people still use herbal medicines, which are mainly made of medicinal plants, while the current estimate is 3.5 billion, or 88% of the world's population (Organization, 2003).

Researchers have successfully identified several phytochemical components present in therapeutic plants due to scientific advancements. These discoveries have been made faster by using *in silico* investigations. These drugs are employed as preventatives due to their various physiological effects (Wangkheirakpam, 2018). Drug design can be rapidly developed and explored using computer-aided drug development (CADD) techniques and molecular docking, as demonstrated by *in silico* methods. Through a successful molecular docking process, the ligands' condition on the binding site and their physical interaction with the protein structure should be determined (Baldi, 2010).

Oxidative stress, caused by free radicals, is the primary contributor to most diseases and ailments. Free radicals and similar chemicals are classified as reactive oxygen species (ROS) because they can cause oxidative alterations inside the cell. Free radicals are highly reactive intermediate chemical entities with unpaired electrons that are produced by the body's chemical reactions and metabolic processes, and antioxidants fight these (E.-Y. Kim et al., 2004). ROS are controlled by endogenous enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. However, excessive production of reactive species, whether from exposure to external oxidants or a breakdown in defense mechanisms, damages proteins, lipids, DNA, and cell structures, raising the risk of more than 30 different disease processes. Mild cognitive impairment (MCI), Alzheimer's disease (AD), and Parkinson's disease (PD) are the most well-known neurodegenerative disorders (Lee et al., 2003). It is well-recognized that antioxidants that scavenge free radicals play a significant role in preventing diseases brought on by free radicals (Yu et al., 2002). Both natural and artificial sources of antioxidants are available. The safety of synthetic antioxidants is not always assured, despite their effectiveness. Antioxidants, which are abundant in medicinal plants, can prevent or postpone the oxidation of lipids or other molecules, and plant-based antioxidants are safe and less harmful (Djeridane et al., 2007).

During infection and tissue damage, inflammation is a vital immune system response that guarantees survival. For proper tissue homeostasis to be maintained, inflammatory reactions are necessary (Shaikh et al., 2016). The metabolism of arachidonic acid plays a significant part in several processes that make up the mechanisms of inflammation (Leelaprakash & Dass, 2011). Many diseases can be caused by cellular inflammation, which can either stimulate cell development into tumors or lead to cell death and organ damage. But anti-inflammatory medications, such as non-steroidal anti-inflammatory medicines (NSAIDs), have detrimental consequences on human health. Scientists have focused on finding safe and effective anti-inflammatory molecules from herbal medicines to address the demand for effective anti-inflammatory treatments that can overcome the crippling adverse effects of currently available medications (Bouyahya et al., 2022).

Pain is a complex concept to accurately define due to its diverse range of definitions, pathophysiological causes, durations, and intensities (Mohammad, Md. Anisul, et al., 2025). This imprecise and unpleasant sensation may be triggered by nociceptive or inflammatory substances. These days, there is a wide variety of drugs that relieve pain by acting as anti-inflammatory and anti-nociceptive substances (Ullah et al., 2014). Generally speaking, analgesics fall into

two categories: morphine and non-morphine. Selective, referred to as opiates, morphine and its derivatives exhibit a central depressive action among other psychotropic qualities. Medications recommended for low-intensity pain include non-morphine derivatives, which have analgesic, antipyretic, or anti-inflammatory properties (Maund et al., 2011). However, these drugs don't always work because of their unfavorable side effects. For example, non-steroidal anti-inflammatory medications and opiates can result in heart problems, ulcers, and high blood pressure (Carter et al., 2014). Therefore, developing better, more effective, and more affordable anti-inflammatory and analgesic drugs with fewer side effects is crucial for human well-being.

Natural therapeutic medicines with a better safety profile are constantly sought after to treat these ailments because manufactured pharmaceuticals often have numerous adverse effects. A member of the Anacardiaceae family, *Mangifera sylvatica* Roxb. is a wild fruit tree species that is Indigenous to Bangladesh, India, Myanmar, Nepal, Thailand, China, and Cambodia (Akhter et al., 2022). Chittagong, Cox's Bazar, Chittagong Hill Tracts, and Sylhet districts are endemic to *M. sylvatica*, also known as jangliam, in Bangladesh (Baul et al., 2016). Evidence of positive medical effects is mounting; for example, a recent study found that the leaves of *M. sylvatica* have thrombolytic qualities that may be able to lyse blood clots (Zaman, Parvez, Hasan, et al., 2015). Additionally, the leaves can be utilized as antidiarrheal medications (Zaman, Parvez, Jakaria, et al., 2015).

Since the pharmacological effects and bioactive phytochemicals of this plant have not been previously investigated, the goal of this study is to evaluate these properties. This study fills a clear research gap in the field of medicinal plant-based pharmaceuticals by objectively demonstrating the therapeutic potential of *M. sylvatica*. Due to this, the ethanolic extract of *M. sylvatica* leaves has been investigated for its analgesic, anti-inflammatory, and antioxidant properties using *in vivo*, *in vitro*, and *in silico* methods.

2 | Materials & Methods

2.1 | Chemicals and Reagents

The chemicals used throughout the experiment include formalin, acetic acid, diclofenac sodium, DPPH, Folin-Ciocalteu reagent, Tween-80, and DMSO, supplied by the International Islamic University Chittagong. Ethanol (Sigma Chemicals, USA) was bought through Taj Scientific Ltd. In this experiment, analytical-grade chemicals were utilized for all other substances.

2.2 | Collection and Identification of the Plant

The leaves of *M. sylvatica* were collected at the end of April 2024 from the Chittagong Hills region, specifically from Khagrachari. The plant was identified and authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh. A voucher specimen of the sample has been stored under the identification code MMA050424-27 in the institution's herbarium, Department of Botany, University of Chittagong.

2.3 | Preparation of Extract

The plant leaves were shade-dried at room temperature and then ground into powder using a mechanical grinder. Five hundred grams of leaf powder were soaked in 2.3 L of analytical-grade ethanol for five days. The mixture was filtered through muslin cloth and then through filter paper to obtain an explicit solvent. The ethanolic filtrate was evaporated at room temperature in a ventilated space, and the concentrated extract was dried for an hour at 40°C. The final dried extract was stored in an amber glass vial at 4°C for future use.

2.4 | Experimental Animals and Ethical Statement

Swiss albino male and female mice, weighing 25–30 g, were gathered from Rajshahi, Bangladesh, at the age of 6–7 weeks. In addition to fresh water and sufficient pelleted food (purchased from a local pet store in Chittagong), the animals were housed in polypropylene boxes. Within the experimental setup, the animals were given seven days to get used to their new surroundings. The animals were maintained at $25 \pm 2^\circ\text{C}$, with a 12-hour light/dark cycle and a relative humidity of 55–65%. This study was approved by the Institutional Review Board of the P&D Committee (Pharm-P&D/198/15-21–198) of the Pharmacy Department at the International Islamic University Chittagong, located in Chittagong, Bangladesh, for animal experimentation and human sample testing. All biological activity testing has been conducted in accordance with the ethical standards outlined in the 2013 Helsinki Declaration. At the end of the experiment, an anesthesia overdose (Ketamine HCl (100 mg/kg) and Xylazine (7.5 mg/kg) through the intraperitoneal route was given to the mice models followed by euthanasia.

2.5 | Gas Chromatography-mass Spectrometry (GC-MS/MS) Analysis

A Shimadzu GC–MS/MS TQ 8040 was used to analyze chemicals extracted from EMS via electron impact ionization. The analysis was performed on a fused silica capillary column, with temperatures and timings adjusted for optimal separation. The system operated at 53.5 kPa, with a flow rate of 11.0 mL/min. The retention times for the compounds were between 0 to 50, and were identified by comparing the mass spectra obtained from the analysis to the reference spectra available in the National Institute of Standards and Technology (NIST) database and Wiley libraries. This comparison allows for the accurate identification of compounds based on their spectral patterns and characteristics. The detector voltage was set to 0.6 kV, the ion source temperature to 230°C, and the total runtime was 39 minutes. Data were collected in Q3 scan mode (m/z 50–600) with a solvent cut time of 3.5 minutes.

2.6 | Experimental Design for Acute Toxicity

Using doses of 2000 and 4000 mg/kg of EMS, the acute oral toxicity test was conducted by the Organization for Economic Cooperation and Development (OECD)18 experimental methodology Guideline 423 (Jiko et al., 2024). Following OECD (2001) guidelines, three groups of animals ($n = 3$) were used. Group I received EMS (2000 mg/kg), Group II received EMS (4000 mg/kg) in 1% Tween 80 with DMSO, and the control group received only the vehicle. Observations

included behavior, reflexes, skin, fur, digestion, motor activity, respiratory patterns, salivation, lacrimation, defecation, urination, convulsions, tremors, cyanosis, hyperemia, hypothermia, and mortality (Zishan et al., 2024).

2.6.1 | Hematological Examination

On the fourteenth day, after an eight-hour fast, animals were euthanized. Blood was collected for hematological analysis with and without EDTA, and vital organs were removed for histopathological study. Using a Sysmex K21 analyzer, blood parameters such as red and white blood cell counts, hemoglobin, platelets, and others were measured and compared between control and plant-treated groups.

2.6.2 | Histopathological Studies

Multiple organs (liver, kidney, lungs, spleen, Heart) of the experimental mice were removed and divided into small sections. These sections were then immersed in a 10% formaldehyde solution overnight for fixation, after which they underwent dehydration. The dehydrated tissue samples were encased in paraffin. Using a microtome, slices measuring 4 µm in thickness were prepared. The slices were treated with xylene to remove the paraffin, rehydrated by passing them through decreasing concentrations of alcohol, and rinsed with distilled water for 5 minutes. The slices were stained with hematoxylin, a basic dye, for 40 seconds, followed by counterstaining with eosin, an acidic dye, for 20 seconds. Once staining was complete, the slides were observed under an OLYMPUS CX43 microscope at magnifications of 400X to detect any signs of damage, such as vascular changes, vacuolar degeneration, loss of cellular structure, and modifications in cell architecture. Notably, no significant architectural alterations were observed in the group exposed to our extract. Images of the slides were captured using an OLYMPUS EP 50 camera (Habbu et al., 2008).

2.7 | Antioxidant Activity

2.7.1 | DPPH Radial Scavenging Activity

The antioxidant activity of the ethanolic extract is examined using a slightly modified version of the Alam et al. approach (Hossen et al., 2022). Plant extract (0.1 mL) was mixed with 3 mL of 0.004% DPPH ethanol solution, incubated for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid served as the positive control, and DPPH in ethanol was the negative control. Each sample was measured thrice and averaged, and the scavenging effect percentage and IC₅₀ value were calculated using a calibration curve:

$$\% \text{ Scavenging Activity} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c is the absorbance of the negative control, and A_s is the absorbance of the test sample

2.7.2 | Reducing Power Assay

EMS's antioxidant activity was assessed using the reducing power assay technique (Oyaizu, 1986). Reduction-potent substances convert ferricyanide (Fe³⁺) to ferrocyanide (Fe²⁺), forming a ferric ferrous complex measured at 700 nm. A mixture of phosphate buffer, potassium ferricyanide, and test sample was incubated, centrifuged, and mixed with ferric chloride. Absorbance was measured at 700 nm, with ascorbic acid as the standard. Higher absorbance indicated greater reducing power (Khanum et al., 2013). The percent increase in reducing power was calculated using the following equation:

$$\% \text{ Increase in reducing power} = \frac{A_t - A_b}{A_b} \times 100$$

Where, A_t is the absorbance of the test solution, and A_b is the absorbance of the blank.

2.8 | Anti-inflammatory Activity

2.8.1 | Inhibition of Protein Denaturation

The inhibition of protein denaturation experiment, which follows the Mizushima and Kobayash method with minor modifications, is used to examine the anti-inflammatory properties of the ethanolic extract (Subhashree et al., 2023). Each reaction mixture contained 2 ml of extract EMS (32.25–500 µg/ml), 2.8 ml PBS, and 0.2 ml egg albumin (5%). The positive control used aspirin. Samples were incubated at 37°C for 15 minutes, then at 70°C for 5 minutes. Absorbance was measured at 660 nm using a UV-visible spectrometer, with triplicate readings averaged. Protein denaturation inhibition was calculated using the following formula:

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

Where, V_t = Absorbance of the test sample, V_c = Absorbance of the control

2.9 | Analgesic Activity

2.9.1 | Acetic acid-induced Writhing Method

Using a slightly modified version of Koster et al.'s methodology, the acetic acid-induced writhing method was used to assess the sample's analgesic effectiveness in mice (Owoyele et al., 2001). Acetic acid was injected intraperitoneally into overnight-fasted mice to induce pain. The animals were divided into five groups of three mice each:

- - Group I (negative control): received distilled water (10 ml/kg).
- - Group II (positive control): received diclofenac sodium (10 mg/kg in 1% Tween 80).
- - Groups III, IV, and V: received EMS extract at 100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively.

Test samples were administered orally 30 minutes before injecting 0.7% acetic acid. Each mouse was observed for writhing (abdominal constriction and hind limb stretching) over 10 minutes, with incomplete writhes counted as half. The percentage of writhing inhibition was calculated using a specific formula.:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

where A = mean number of writhing in the control group and

B = mean number of writhing in the test group.

2.9.2 | Formalin-induced Paw Licking Test

The approach used was comparable to that which has already been published (Shibata et al., 1989). Mice were divided into five groups of three, treated with distilled water (1 ml/kg, i.p.), EMS (100/200/400 mg/kg, s.c.), or diclofenac sodium (10 mg/kg, s.c.). After 30 minutes, 50 µL of 0.6% formalin was injected into the left hind paw. Pain response (licking/biting time) was observed for 30 minutes, with anti-nociceptive effects measured in two phases: early phase (0 to 5 minutes post-injection) and late phase (20–30 minutes post-injection).

% of inhibition = (MLc – MLt)/MLc

Here

MLc = Mean licking time in control

MLt = Mean licking time in the test.

2.10 | In Silico Study

2.10.1 | Software Tools

The Protein Data Bank (PDB), Swiss PDBViewer, AutoDockVina, DrugBank, PubChem, PyMOL, and Discovery Studio Visualizer 2021 (BIOVIA) were among the tools and resources used in the research (Mohammad, Chowdhury, et al., 2025).

2.10.2 | ADME/T Study

The online tools AdmetSAR (<http://lmmd.ecust.edu.cn/admetSar1/predict/>) and pKCSM (<http://biosig.unimelb.edu.au/pkcsM/>) were utilized to predict the toxicological and ADME/T (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of the newly discovered compounds, as drug toxicity is a significant concern. SwissADME and the Lipinski rule of five were used to screen the chemicals on that list (Ali et al., 2024).

2.10.3 | Selection of Ligands

From the PubChem Database, 24 chemicals chosen for the final GC-MS/MS analysis were obtained in 3D SDF format. Compounds with a 2D SDF format whose 3D conformer wasn't available from the PubChem Database were converted to 3D SDF format using Open Babel (O'Boyle et al., 2011). The MMFF94 force field was utilized by the PyRx program to lower the ligand's energy before doing molecular docking studies (Dallakyan & Olson, 2015) (Eberhardt et al., 2021).

2.10.4 | Validation of the Ligands

The physical and molecular properties of these compounds, along with pharmacokinetic elements such as ADME/T (absorption, distribution, metabolism, excretion, and toxicity), significantly impact the choice of these chemicals as potential treatments. Using the pKCSM online tool (<http://biosig.unimelb.edu.au/pkcsM/>), the listed compounds' potential as ligands against therapeutic targets was confirmed on July 31, 2021 (Pires et al., 2015). The compounds were then evaluated for drug potential utilizing Lipinski's rule of five and the SwissADME web server (Daina et al., 2017) (Mohammad, Hossain, et al., 2025).

2.10.5 | Protein Preparation

The RCSB protein data bank provided the crystal structures of the human cytochrome P450 CYP2C9 (PDB: 1OG5), human cyclooxygenase-2 (PDB: 5IKR), and cyclooxygenase-2 inhibitor (PDB: 6COX) for their antioxidant, anti-inflammatory, and analgesic qualities, respectively. Data previously published by Kurumbail et al. were used to identify the active location of the enzyme (Kurumbail et al., 1996). The required cleaning and preparations, including the elimination of water, cofactors, and heteroatoms, were carried out using Swiss-PdbViewer (v4.1) and the BIOVIA Discovery Studio 4.5 Client (Mohammad, Mamun, et al., 2025). The PyRx-virtual screening tool and the force field of MMFF94 were utilized to reduce the protein after hydrogen atoms were added to the target protein's structure (Mohammad, Rasel, et al., 2025). The target protein was retained in PDB format to facilitate docking experiments.

2.10.6 | Molecular Docking and Post-Docking Analysis

The docking calculations were performed using PyRx 0.3 (<http://pyrx.scripps.edu>) (accessed July 31, 2021) and AutoDock, version 4.2 (Saddala et al., 2013). A grid box with the following dimensions was created using AutoGrid: X: 51.0689; Y: 39.0321; Z: 23.9632 Å points; and the grid spacing was 0.375 Å. PyMOL

was used to analyze the docking results. These technologies can be used to identify the type of contact (e.g., cation- π , hydrogen bond, or π - π interactions) and how it contributes to ligand binding. PyMOL (Protein–ligand docking: Current state and future challenges) was used to collect further information on the interaction between ligands and receptors (Hossain et al., 2025).

2.11 | Statistical Analysis

The standard error of the mean, or Mean $\pm$ SEM, was used to present the data. The statistical software "Statistical Package for the Social Sciences" (SPSS, Version 16.0, IBM Corporation, New York) was used to conduct the statistical analysis. Post hoc Dunnett test was used for comparisons following a one-way analysis of variance (ANOVA). The significance levels were determined using the following criteria: *p < 0.05, **p < 0.01, and ***p < 0.001. When compared to the study group, these numbers demonstrate statistical significance.

3 | Results

3.1 | GC-MS/MS Analysis

The GC-MS/MS analysis of EMS identified approximately 60 compounds, with peak areas ranging from 0.15% to 8.29%, revealing a diverse chemical profile dominated by key compounds such as Squalene (13.91%), Isopropyl myristate (12.29%), and various triterpenoids and sterols like D-Friedoolean-14-en-3-one (6.47%), Lup-20(29)-en-3-one (5.97%), and γ -Sitosterol (3.0%). Other notable constituents include Vitamin E (2.15%) and Lupeol (2.14%), while the remaining compounds each contribute less than 2% of the total peak area (Table 1 and Fig. 1). This complex mixture, rich in bioactive terpenoids, sterols, and fatty acid derivatives, suggests potential applications in pharmaceuticals.

3.2 | Acute Oral Toxicity

3.2.1 | General Sign and Behavioral Interpretation

The acute toxic effects of the ethanolic extract were assessed under OECD Guideline 423, using a limit test dose of 4,000 mg/kg. No treatment-related toxic symptoms or mortality were observed after oral administration of the plant extract at doses of 2,000 and 4,000 mg/kg. The general behavior of the treated animals and the control group was monitored initially for a short period (4 hours) and then for a longer period (14 days). No drug-related changes were noted in behavior, breathing, skin condition, water consumption, food intake, or body temperature. Consequently, the extract appears to be safe at a dose of 4,000 mg/kg, with an estimated median lethal dose (LD₅₀) more than 4,000 mg/kg. However, mild jerking movements were noted in a few male mice in the 4000 mg/kg treated group compared to the control group, which subsided after 20 minutes of dose administration. No additional indicators of potential toxicity, including piloerection, tremors, convulsions, piloerection, diarrhoea, or significant alterations in food and drink consumption, were observed. The parameters recorded for the acute toxicity study, comparing the test group with the normal group, are presented in Table 2.

Table 2
General appearance and behavioral observations of the acute toxicity study for the control and treated groups.

Observation	Control		2,000 mg/kg		4,000 mg/kg	
	30mins	4hrs	30mins	4Hrs	30mins	4Hrs
Digestion	No	No	No	No	No	No
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Temperature	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	No effect	No effect	No effect	No effect
Food intake	Normal	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal	Normal
Rate of respiration	Normal	Normal	No effect	No effect	No effect	No effect
Change in skin	No effect	No effect	No effect	No effect	No effect	No effect
Sedation	No effect	No effect	Mild Sedation	Mild Sedation	Mild Sedation	Mild Sedation
Eye color	No effect	No effect	No effect	No effect	No effect	No effect
Diarrhea	Not present	Not present	Not present	Not present	Not present	Not present
Tremor	Not present	Not present	Not present	Not present	Not present	Not present
General physique	Normal	Normal	Normal	Normal	Mild Lethargy	Mild Lethargy
Coma	Not present	Not present	Not present	Not present	Not present	Not present
Death	Alive	Alive	Alive	Alive	Alive	Alive

3.2.2 | Effect of Plant Extract on Organ Body Weight

No significant differences in average organ weights were observed between control and extract-treated groups at 2000 and 4000 mg/kg doses (Table 3, Fig. 2). While mild weight variations were noted at 2000 mg/kg, the 4000 mg/kg dose of EMS caused significant changes. The results indicate that the extract has adverse effects on vital organs (liver, kidney, heart, pancreas, and small intestine) at the higher dose. Statistically significant differences were found in average organ weights between the extract-treated and control groups.

Table 3
Effect of oral administration of ethanolic extract of *M. sylvatica* on average organ weight (g) of mice.

Organ	Organ Weight		
	Control	2000 mg/kg extract	4000 mg/kg extract
Liver	5.44 ± 0.35	6.71 ± 0.89*	7.3 ± 0.68**
Lungs	0.91 ± 0.09	1.09 ± 0.17*	1.29 ± 0.18**
Kidney	1.47 ± 0.1	2.16 ± 0.27**	2.71 ± 0.35**
Heart	0.67 ± 0.07	0.98 ± 0.86**	1.27 ± 0.13***
Spleen	0.47 ± 0.04	0.67 ± 0.091*	0.9 ± 0.085***
Note: Values are represented as Mean ± SEM (n = 3). *P < 0.05, **p < 0.01, ***p < 0.001 when compared to control group.			

3.2.3 | Effect of Plant Extract on Hematological Parameters.

Hematological test results are summarized in Table 4. All tested parameters (total blood count, hemoglobin, red blood cells, total white blood cells, monocytes, lymphocytes, and packed cell volume) remained within normal limits compared to the control group. However, significant changes (p < 0.001) in platelet and neutrophil counts were observed at both 2000 and 4000 mg/kg doses. No toxicologically substantial differences were found between the extract-treated animals and the controls.

Table 4
Effect of oral *M. sylvatica* extract on hematological parameters.

Parameter	Normal Control	2000 mg/kg extract	4000 mg/kg extract
WBC [10 ³ /uL]	3.23 ± 0.40	6.86 ± 0.54***	7.7 ± 0.58***
RBC [10 ⁶ /uL]	7.87 ± 0.53	8.13 ± 0.53***	9.24 ± 0.70***
Hemoglobin (g/dL)	11.4 ± 0.51	12.6 ± 0.72*	13.2 ± 0.65**
HCT (%)	40.9 ± 0.86	43.7 ± 1.15**	50.2 ± 0.80***
MCV (fL)	52 ± 0.70	55 ± .69***	57.4 ± 0.66***
MCH (pg)	14.5 ± 0.90	13.8 ± 0.63*	16.7 ± 0.74***
MCHC (g/dL)	27.9 ± 0.75	25.1 ± 0.53***	29.1 ± 0.78***
PLT [10 ³ /uL]	320 ± 0.92	851 ± 0.65***	769 ± 0.85***
RDW-SD (fL)	27 ± .79	39.7 ± 0.64***	35 ± 0.59***
RDW-CV (%)	17.7 ± 0.60	23.2 ± 0.68***	16.4 ± 0.61***
PDW (fL)	7 ± 0.61	6.9 ± 0.59**	21 ± 0.70***
MPV (fL)	7.1 ± 0.70	7 ± 0.72	8.2 ± 0.73**
P-LCR (%)	6.9 ± 0.78	6.7 ± 0.91	11.1 ± 0.71***
PCT (%)	0.23 ± 0.01	0.59 ± 0.02***	0.628 ± 0.03***
NEUTROPHIL (%)	1.11 ± 0.11	22.5 ± 0.69***	24.4 ± 0.71***
LYMPHOCYTE	1.56 ± 0.03	0.6 ± 0.12***	0.7 ± 0.07***
MONOCYTE (%)	0.14 ± 0.09	0.6 ± 0.04***	2.1 ± 0.34**
BASOPHIL (%)	0.29 ± 0.02	8.2 ± 0.53***	6.1 ± 0.65***
IG	0.02 ± 0.0005	0.9 ± 0.07***	0.14 ± 0.65***

3.2.3 | Histopathological Study

The histological analysis of the treated organs is presented in Fig. 3. No significant changes were observed in the monocytes, blood vessels, or myocardium of the heart. Similarly, in the kidneys and liver, there were no notable alterations in the glomeruli or central veins. Additionally, the lung's pulmonary vessels, bronchioles, and alveoli, as well as the spleen's red and white pulp, remained unaffected.

3.3.1 | DPPH Radical Scavenging Assay

In the DPPH assay, the EMS extract demonstrated notable antioxidant activity, albeit lower than that of the standard ascorbic acid. EMS exhibited a dose-dependent inhibition ranging from 5.25% to 58.39%, with an IC₅₀ value of 348.72 µg/mL, indicating significant free radical scavenging potential. In comparison, ascorbic acid showed superior antioxidant activity, achieving a maximum inhibition of 67.29% at 1000 µg/mL and a lower IC₅₀ value of 263.5 µg/mL, reflecting its higher efficacy. These findings, presented in Table 5 and Fig. 4, suggest that while EMS possesses significant antioxidant properties, it is less potent than ascorbic acid, highlighting its potential as a natural source of antioxidants for various applications.

Table 5
Evaluation of Antioxidant Activity of EMS through DPPH Radical Scavenging Assay.

Concentration (µg/mL)	EMS		Ascorbic Acid	
	% Inhibition	IC ₅₀	% Inhibition	IC ₅₀
3.9	5.25	348.72	6.85	263.5
7.81	7.81		11.01	
15.62	14.34		19.97	
31.25	18.44		27.01	
62.5	28.23		38.54	
125	37.26		48.78	
250	47.5		56.98	
500	58.39		67.29	

3.3.2 | Reducing Power Assay

The reductive ability of EMS was assessed by monitoring the transformation of Fe³⁺ to Fe²⁺ in a concentration-dependent manner, with results showing a significant increase in reducing power as the EMS concentration increased (p < 0.001). This activity was comparable to the standard ascorbic acid, although EMS exhibited lower potency. The concentration-dependent trend observed in this assay aligns with the results of the antioxidant activity, further confirming the extract's potential as a natural reducing agent. These findings, illustrated in Fig. 5, highlight the promising role of EMS in applications that require redox-active properties. The IC₅₀ comparison of both test results are illustrated in Fig. 6.

3.4 | Anti-Inflammatory Activity

3.4.1 | Protein Denaturation Assay

The in vitro anti-inflammatory activity of EMS was evaluated by measuring its ability to inhibit protein denaturation, demonstrating a concentration-dependent response with mean inhibition percentages of 77.5%, 67.65%, 54.41%, 39.7%, and 33.82% at concentrations of 500, 250, 125, 62.5, and 31.25 µg/mL, respectively. Although the standard diclofenac sodium exhibited superior efficacy, with a maximum inhibition of 86.76% at 1000 µg/mL and a lower IC₅₀ value of 36.81 µg/mL, EMS still showed significant anti-inflammatory potential, as evidenced by its IC₅₀ value of 142.52 µg/mL. These findings, presented in Fig. 7, suggest that EMS possesses notable anti-inflammatory properties, making it a promising candidate for further investigation in the development of natural anti-inflammatory agents.

3.5 | Analgesic Activity

3.5.1 Acetic Acid-Induced Writhing Method

The effects of EMS on acetic acid-induced writhing in experimental mice, as shown in Table 6 and Fig. 8, revealed a significant reduction (p < 0.01) in writhing episodes in a dose-dependent manner following intraperitoneal administration. Oral administration of EMS at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight resulted in percent inhibitions of 23.48%, 38.78%, and 59.20%, respectively, demonstrating its potential analgesic activity. However, the standard drug, diclofenac sodium, exhibited superior efficacy, with a percent inhibition of 82.64%, highlighting its greater potency. These findings indicate that EMS possesses notable analgesic properties.

Table 6
Evaluation of Analgesic Activity of EMS through Acetic Acid-induced Writhing Test.

Treatment	No of Writhing	% Inhibition
Control	32.67 ± 1.20	-
Diclofenac Na	5.67 ± 0.33***	82.64
EMS 100	25 ± 1.53**	23.48
EMS 200	20 ± 2.31**	32.76
EMS 400	13.33 ± 0.88**	51.8
Note: Values are Mean ± SEM, (n = 3); **p < 0.01, and ***p < 0.001 as compared to vehicle control (one-way ANOVA followed by Dunnett's test).		

3.5.2 | Formalin-Induced Paw Licking Test

EMS demonstrated significant antinociceptive effects in the formalin-induced pain test in mice, suppressing licking activity in both phases of the test in a dose-dependent manner. At a higher dose of 400 mg/kg body weight (p.o.), EMS exhibited substantial inhibition of licking activity (46.03%, and 49.98% in the early and late phases, respectively). In contrast, at 400 mg/kg, it showed comparable efficacy to the standard drug diclofenac sodium against both the early and late phases of formalin-induced pain. These results, summarized in Table 7 and Fig. 9, suggest that EMS possesses notable analgesic properties, with potential activity against both neurogenic and inflammatory pain pathways.

Table 7
Evaluation of Analgesic Activity of EMS through Formalin-induced Paw Licking Test.

Dose	Licking Time (sec)			
	Early Phase		Late Phase	
	Mean ± SEM	% Inhibition	Mean ± SEM	% Inhibition
Control	25.33 ± 2.03	-	27.33 ± 1.20	-
Diclofenac Na	9.72 ± 1.07**	61.63%	6.5 ± 0.33**	76.22%
EMS 100	24.67 ± 1.20	2.61%	24.33 ± 0.67	10.98%
EMS 200	20.33 ± 2.19	19.74%	21.33 ± 0.88*	21.95%
EMS 400	13.67 ± 2.19**	46.03%	13.67 ± 0.88***	49.98%
Note: Values are Mean ± SEM, (n = 3); *p < 0.05, **p < 0.01, and ***p < 0.001 as compared to vehicle control (one-way ANOVA followed by Dunnett's test).				

3.6 | In Silico Study

3.6.1 | ADME/T and Drug-Likeness Analysis

The therapeutic potential of reported phytochemicals from EMS was evaluated by analyzing their pharmacokinetic and drug-likeness properties before docking analysis. Table 8 presents the pharmacokinetic profiles of these compounds. Based on these profiles, it can be inferred that the phytochemicals are unlikely to cause mutagenesis or carcinogenesis and meet all criteria of the Lipinski rule. Significant outcomes were obtained from in silico ADME/T and drug similarity analyses of the identified substances using the pKCSM and SwissADME platforms.

Table 8
ADME/T and drug likeliness study of selected compounds of EMS.

Compounds Name	Absorption		Distribution		Metabolism	Excretion	Toxicity		Drug Likelihood	Bioavailability
	Water Solubility (log mol/L)	Intestinal Absorption (Human) (% Absorbed)	VDss (Human) (log L/kg)	BBB Permeability (log BB)	CYP3A4 Substrate	Total Clearance (log ml/min/kg)	AMES Toxicity	Hepatotoxicity		
Squalene	-8.401	89.002	0.35	0.965	Yes	1.791	No	No	Yes	0.55
Isopropyl myristate	-6.234	92.514	0.128	0.734	Yes	1.773	No	No	Yes	0.55
24-Noroleana-3,12-diene	-6.85	96.674	0.44	0.848	Yes	0.073	No	No	Yes	0.55
Friedoolean-14-en-3-one	-6.493	95.646	0.082	0.687	Yes	-0.132	No	No	Yes	0.55
Lup-20(29)-en-3-one	-3.667	100	-1.315	0.103	No	-1.414	Yes	No	Yes	0.55
Lup-20(29)-en-3-ol, acetate,	-6.006	97.894	-0.12	0.644	Yes	0.06	No	No	Yes	0.55
.beta.-Sitosterol acetate	-3.618	83.456	-1.011	0.282	No	-1.426	Yes	No	Yes	0.55
.gamma.-Sitosterol	-6.773	94.464	0.193	0.781	Yes	0.628	No	No	Yes	0.55
Lupeol	-5.861	95.782	0	0.726	Yes	0.153	No	No	Yes	0.55
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-7.554	90.71	0.468	0.806	Yes	1.686	No	No	Yes	0.55
Cholest-5-en-3-ol, carbonochloridate	-7.074	93.524	0.08	0.676	Yes	-0.335	No	No	Yes	0.55
6-Octadecenoic acid, methyl ester,	-7.436	92.154	0.299	0.777	Yes	1.981	No	No	Yes	0.55
Stigmasta-5,22-dien-3-ol, acetate,	-6.859	97.083	0.051	0.739	Yes	0.539	No	No	Yes	0.55
1-Dodecanol, 3,7,11-trimethyl-	-5.923	91.753	0.424	0.747	No	1.526	No	No	Yes	0.55
Pregn-5-en-20-one, 3,21-bis(acetyloxy)	-4.93	98.706	-0.082	-0.316	Yes	0.552	No	No	Yes	0.55
9-Octadecenamide,	-7.074	90.218	0.281	-0.389	Yes	1.959	No	No	Yes	0.55
Stigmasterol	-6.682	94.97	0.178	0.771	Yes	0.618	No	No	Yes	0.55
Methyl 9,11-octadecadienoate	-7.343	92.66	0.272	0.767	Yes	2.028	No	No	Yes	0.55
8,10-Hexadecadien-1-ol	-6.615	90.816	0.419	0.778	Yes	1.967	No	No	Yes	0.55
Ergosta-5,22-dien-3-ol,	-6.974	95.05	0.407	0.764	Yes	0.57	No	No	Yes	0.55
Phytol	-4.93	98.706	-0.082	-0.316	Yes	0.552	No	No	Yes	0.55
γ-Tocopherol	-7.602	90.043	0.732	0.739	Yes	0.821	No	No	Yes	0.55
Phenol, 4-(methoxymethyl)	-1.244	93.53	0.245	-0.205	No	0.309	No	No	Yes	0.55
9,19-Cyclolanost-24-en-3-ol, acetate,	-5.884	97.36	-0.194	0.711	Yes	0.171	No	No	Yes	0.55

3.6.2 | Molecular Docking Study

Details of the docking analysis results for analgesic, anti-inflammatory, and antioxidant properties are given in Table 9.

Table 9

Binding scores of the chosen compounds from the EMS for antioxidant, anti-inflammatory, and analgesic activity against the human cytochrome P450 CYP2C9 (PDB: 1OG5), human cyclooxygenase-2 (PDB ID: 5IKR), and cyclooxygenase-2 inhibitor (PDB ID: 6COX), respectively.

Compounds	PubChem ID	Docking Score (Kcal/mol)		
		Antioxidant (1og5)	Anti-inflammatory (5lkr)	Analgesic (6Cox)
Squalene	638072	-7.9	-8	-8.4
Isopropyl myristate	8042	-5.2	-6.5	-6.6
24-Noroleana-3,12-diene	15427754	-10.8	-0.8	-8.7
D-Friedoolean-14-en-3-one	92785	-9.8	-0.5	-8.3
Lup-20(29)-en-3-one	92158	-10.1	-0.3	-7.8
Lup-20(29)-en-3-ol, acetate,	92157	-10.6	-0.5	-8.2
beta.-Sitosterol acetate	5354503	-9.2	-4.6	-7.1
gamma.-Sitosterol	457801	-9.4	-5.3	-7.2
Lupeol	259846	-10.7	-0.2	-7.6
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	5366244	-6.2	-6.2	-7.2
Cholest-5-en-3-ol,carbonochloridate	111262	-9.1	-4.7	-7
6-Octadecenoic acid, methyl ester,	5362717	-5.8	-6.4	-6.8
Stigmasta-5,22-dien-3-ol, acetate,	6432445	-9.9	-4.4	-7.9
1-Dodecanol, 3,7,11-trimethyl	138824	-5.8	-6.5	-6.7
Pregn-5-en-20-one, 3,21-bis(acetyloxy)	99488	-8.8	-5.2	-7.9
9-Octadecenamide	5283387	-5.8	-6.4	-7
Stigmasterol	5280794	-9	-5.7	-7.5
Methyl 9,11-trans-octadecadienoate	11748436	-5.6	-6.8	-7
8,10-Hexadecadien-1-ol	5364617	-5.8	-6.6	-6.5
Ergosta-5,22-dien-3-ol,	6432458	-10.2	-5.3	-7.4
Phytol	5280435	-6.6	-6.5	-7.3
γ-Tocopherol	92729	-8.6	-8.3	-7.9
Phenol, 4-(methoxymethyl)	79310	-5.2	-5.8	-5.6
9,19-Cyclolanost-24-en-3-ol, acetate,	518616	-10.4	-2.6	-6.6
Standards (Ascorbic acid, Diclofenac Na)		-5.3	-8.1	-8.4

3.6.2.1 | Docking Study for Antioxidant Activity

This study identified potential antioxidant compounds by screening them against human cytochrome P450 CYP2C9 (PDB ID: 1OG5). The compounds from the EMS extract exhibited binding affinities ranging from -10.8 to -5.2 kcal/mol for this protein. Notably, three compounds—24-Noroleana-3,12-diene, Lupeol, and Lup-20(29)-en-3-ol, acetate showed superior binding affinities of -10.8, -10.7, and -10.6 kcal/mol, respectively, surpassing the conventional inhibitor Ascorbic Acid, which had a binding affinity of -5.3 kcal/mol. The top-performing compound, 24-Noroleana-3,12-diene, formed eleven hydrophobic interactions with key amino acid residues in the active site, including ILE205 (2), LEU208 (2), ALA477, LEU366, PRO367, PHE100 (2), and Phe476 (2), as detailed in Table 10 (Section 1) and illustrated in Fig. 10.

Table 10

EMS's selected phytochemicals *in silico* binding affinity and non-bonding interaction for antioxidant, anti-inflammatory, and analgesic properties, respectively.

Section number	Receptor	Compound	Binding Affinity (kcal/mol)	Bond type	Amino acids
1	1og5	24-Noroleana-3,12-diene	-10.8	Alkyl	ILE205, LEU208, ALA477, LEU366, PRO367
				Pi-Alkyl	PHE100, PHE476
		Lupeol	-10.7	Conventional Hydrogen Bond	GLY296
				Alkyl	ALA103, LEU208, PRO36, ILE205, ARG97, VAL113, LEU366, ILE99
				Pi-Alkyl	PHE100, PHE114
		Lup-20(29)-en-3-ol, acetate,	-10.6	Alkyl	ALA103, LEU208, PRO367, ILE205, VAL113, LEU366, ILE99
				Pi-Alkyl	PHE100, PHE114
		Ascorbic acid (Standard)	-5.3	Conventional Hydrogen Bond	ARG97, LEU366, ARG433, SER365
				Carbon Hydrogen Bond	PHE428, SER429, CYS435, ARG433
2	5ikr	γ -Tocopherol	-8.3	Conventional Hydrogen Bond	ARG120
				Carbon Hydrogen Bond	LYS83
				Alkyl	VAL116, ARG120, VAL349, ALA527, PRO86, VAL89, LEU93, VAL116, VAL349, LEU359, LEU531, LEU352
				Pi-Alkyl	TYR355, TRP387, VAL89
		Squalene	-8	Alkyl	PRO86, VAL89, VAL116, ARG120, VAL349, ALA527, VAL89, LEU93, VAL116, VAL89, ARG120, VAL116, VAL349, LEU359, LEU531, LEU352, VAL349, LEU384, LEU352, MET522
				Pi-Alkyl	TYR115, TYR355, TYR385, TRP387, PHE518
		Methyl 9,11-octadecadienoate	-6.8	Conventional Hydrogen Bond	SER353
				Alkyl	VAL344, VAL349, VAL523, ALA527, LEU352, VAL349, VAL228, LEU534
				Pi-Alkyl	PHE205, PHE205, PHE209, TYR348, TYR355, TYR385, TRP387
		Diclofenac Na (Standard)	-8.1	Conventional Hydrogen Bond	TYR385
				Carbon Hydrogen Bond	SER530
				Pi-Alkyl	LEU352, ALA527, VAL349, ALA527, LEU531
3	6cox	24-Noroleana-3,12-diene	-8.7	Alkyl	ALA516
				Pi-Alkyl	HIS351
		Squalene	-8.4	Alkyl	VAL116, VAL349, VAL523, ALA527, LEU359, LEU531, LEU93, ILE92, ILE112, LEU352, LEU384, MET522, VAL349
				Pi-Alkyl	TRP100, TYR115, TYR348, TYR355, TYR385, TRP387, PHE518
		Ascorbic acid (Standard)	-8.3	Alkyl	LEU352
				Pi-Alkyl	HIS356
				Pi-Pi T-shaped	TRP387
		Diclofenac Na (Standard)	-8.4	Pi-Alkyl	VAL349, VAL523, ALA527

3.6.2.2 | Docking Study for Anti-Inflammatory Activity

In addition, this study explored potential anti-inflammatory compounds by screening them against human Cyclooxygenase-2 (PDB ID: 5IKR). The compounds derived from the EMS extract demonstrated binding affinities ranging from - 8.3 to -0.3 kcal/mol for this protein. Notably, γ -Tocopherol, Squalene, and Methyl 9,11-octadecadienoate exhibited binding affinities of -8.3, -8.0, and - 6.8 kcal/mol, respectively, which are comparable to the standard inhibitor Diclofenac Na

(-8.1 kcal/mol). γ -Tocopherol, the most potent compound, formed 23 hydrophobic interactions with amino acid residues in the target receptor’s active site. Additionally, it engaged in one hydrogen bond and one electrostatic interaction with Arg120 [Table 10 (Section 2), Fig. 11].

3.6.2.3 | Docking Study for Analgesic Activity

In this study, potential analgesic compounds were screened using the Cyclooxygenase-2 inhibitor (PDB ID: 6COX). The compounds from the EMS extract showed binding affinities ranging from - 8.7 to -5.6 kcal/mol for this protein. Notably, 24-Noroleana-3,12-diene, Squalene, and D-Friedoolean-14-en-3-one exhibited binding affinities of -8.7, -8.4, and - 8.3 kcal/mol, respectively, which are comparable to the conventional inhibitor Diclofenac Na (-8.4 kcal/mol). The top-performing compound, 24-Noroleana-3,12-diene, formed three hydrophobic interactions with two active site amino acid residues: ALA516 and HIS351 (2) [Table 10 (Section 3), Fig. 12].

3.7 | Pass Prediction

Twenty-four carefully selected EMS compounds were evaluated for their antioxidant, anti-inflammatory, and analgesic properties using the PASS online tool. The results indicated that substances with significant molecular potency had Pa values higher than Pi (Table 11).

Table 11
PASS Prediction of the selected biologically active Compounds of EMS.

Compound Name	Biological Activity					
	Antioxidant		Anti-inflammatory		Analgesic	
	Pa	Pi	Pa	Pi	Pa	Pi
Squalene	0,657	0,004	0,701	0,016	0,474	0,053
Isopropyl myristate	0,227	0,043	0,446	0,074	0,446	0,073
24-Noroleana-3,12-diene	0,280	0,027	0,848	0,005	0,726	0,003
Friedoolean-14-en-3-one	0,209	0,050	0,842	0,005	0,644	0,005
Lup-20(29)-en-3-one	0,255	0,034	0,767	0,009	0,605	0,007
Lup-20(29)-en-3-ol, acetate, .beta.-Sitosterol acetate	0,300	0,023	0,737	0,012	0,679	0,004
.gamma.-Sitosterol	0,171	0,078	0,575	0,037	0,517	0,028
Lupeol	0,178	0,072	0,467	0,067	0,558	0,014
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0,280	0,027	0,708	0,015	0,726	0,003
Cholest-5-en-3-ol, carbonochloridate	0,475	0,008	0,458	0,070	0,300	0,182
6-Octadecenoic acid, methyl ester,	0,131	0,126	0,287	0,174	0,441	0,077
Stigmasta-5,22-dien-3-ol, acetate,	0,269	0,030	0,607	0,030	0,573	0,011
1-Dodecanol, 3,7,11-trimethyl	0,203	0,053	0,546	0,044	0,559	0,013
Pregn-5-en-20-one, 3,21-bis(acetyloxy)	0,419	0,010	0,406	0,093	0,452	0,069
9-Octadecenamide	0,188	0,063	0,826	0,005	0,589	0,009
Stigmasterol	0,167	0,082	0,384	0,104	0,598	0,008
Methyl 9,11-octadecadienoate	0,215	0,048	0,542	0,045	0,601	0,008
8,10-Hexadecadien-1-ol	0,329	0,019	0,664	0,020	0,552	0,015
Ergosta-5,22-dien-3-ol,	0,362	0,016	0,678	0,019	0,523	0,025
Phytol	0,276	0,028	0,561	0,040	0,636	0,005
γ -Tocopherol	0,475	0,008	0,458	0,070	0,300	0,182
Phenol, 4-(methoxymethyl)	0,927	0,003	0,775	0,008	-	-
9,19-Cyclolanost-24-en-3-ol, acetate,	0,271	0,030	0,345	0,126	0,481	0,048
	0,329	0,019	0,698	0,016	0,519	0,026

4 | Discussion

Medicinal plants represent the most potent source of novel bioactive molecules for developing new therapies (S. Alam et al., 2020). Consequently, plant-based treatments are widely utilized in developing countries and are highly valued for their positive impacts on human health. In these regions, approximately 80% of patients rely on traditional medicines (H.-S. Kim, 2005). Despite the availability of various treatment modalities for numerous diseases, achieving complete alleviation of symptoms without side effects remains a challenge. As a result, the safety, efficacy, onset and duration of action, and potential side effects of current drugs have become critical concerns, driving the demand for new therapeutic options. Given the diversity of neural targets, herbal medicine

holds significant promise for addressing these challenges (Fajemiroye et al., 2016). Therefore, this study aimed to assess the acute toxicity, antioxidant, anti-inflammatory, and analgesic properties of the ethanolic extract of *M. sylvatica*.

The current study aimed to assess the acute toxicity of the ethanol leaf extract of *M. sylvatica* and determine a safe dosage range for further research. The acute oral toxicity was evaluated in swiss albino mice at single doses of 2,000 and 4,000 mg/kg body weight. The animals were closely monitored for the first 4 hours, followed by a 72-hour observation period for any signs of toxicity. No significant behavioral changes or mortality were noted across all groups, although sedation and mild jerking movements were observed at dose levels of 4,000 mg/kg. The extract appeared to be safe at 4,000 mg/kg, and the LD₅₀ of the EMS is estimated to be greater than 4,000 mg/kg. Compounds with an oral LD₅₀ exceeding 1,000 mg/kg are generally considered low in toxicity (Adeneye & Olagunju, 2009), suggesting that the ethanolic extract of *M. sylvatica* is practically non-toxic at a single dose of 4,000 mg/kg. However, for chronic conditions like cancer, diabetes, or hyperlipidemia, where multiple doses may be required, the safety of the extract on organ weight, hematological, and biochemical parameters needs further investigation through a sub-acute toxicity study. This study was conducted using the same doses (2,000 and 4,000 mg/kg) following OECD guidelines (Kunimatsu et al., 2004). Changes in body weight are often linked to the toxic effects of chemicals or drugs; however, scientific evidence suggests that such fluctuations may also result from fat accumulation or physiological adaptations to plant extracts, rather than direct toxicity (Arsad et al., 2013). In conclusion, the single-dose acute toxicity study demonstrated that the extract is safe up to a dose of 4,000 mg/kg. The study evaluated the antioxidant activity of the ethanolic extract of *M. sylvatica* using the DPPH free radical scavenging assay and the reducing power assay. At 500 µg/mL, EMS showed 58.39% scavenging activity, compared to 67.29% for ascorbic acid (AA). Increased concentrations of both EMS and AA enhanced scavenging effects, indicating that higher metabolite levels promote DPPH-H bond formation, leading to DPPH discoloration from purple to yellow due to reduced absorbance and increased inhibition (Rohmah et al., 2020). Flavonoids, triterpenes, and tannins are examples of phenolic chemicals found in an extract that function as electron donors and provide DPPH electrons, discoloring it purple (Fatema et al., 2024). According to multiple accounts, this color shift signifies the presence of antioxidant activity in plant extract (Baliyan et al., 2022). The reducing power assay confirmed EMS's antioxidant potential, attributed to reductones that break free radical chains by donating hydrogen atoms (Tanaka et al., 1988). Because a substance can lower ROS by donating hydrogen atoms, it can operate as an antioxidant (Jayaprakasha et al., 2001). Therefore, the extract EMS's ferric-reducing capability suggests that it can donate hydrogen atoms in a dose-dependent way. Phenolic compounds also enhance cellular antioxidant systems, increasing glutathione levels by approximately 50% (B. Alam et al., 2013). Recent studies have shown that two varieties of *Mangifera indica* leaves exhibit significant antioxidant activity in DPPH and reducing power assays (Sultana et al., 2012) (Mohan et al., 2013). GC-MS/MS analysis identified terpenoids such as 24-Noroleana-3,12-diene, Neophytadiene, Lupeol, Caryophyllene, Betulinaldehyde, and Phytol in EMS, which likely contribute to its antioxidant properties. These findings underscore the potential of EMS as a natural source of antioxidants with therapeutic benefits.

The anti-inflammatory potential of the ethanolic extract of *M. sylvatica* was assessed using a protein denaturation assay. Protein denaturation is a known contributor to inflammation and may lead to the production of autoantigens in inflammatory diseases. The denaturation process likely involves disruptions in electrostatic, hydrogen, hydrophobic, and disulfide bonds (Bagad et al., 2011). The results indicate that EMS effectively inhibits protein denaturation, potentially controlling the production of autoantigens. Its activity was comparable to the standard drug diclofenac sodium. Terpenoids, known for their significant analgesic and anti-inflammatory properties, are likely responsible for the observed effects (Neukirch et al., 2005) (Moody et al., 2006). Previously, *M. indica* has demonstrated significant anti-inflammatory effects in both in vitro and in vivo studies (Garrido et al., 2004). Specific terpenoids identified in EMS, such as 24-Noroleana-3,12-diene, Beta-Carotene, Neophytadiene, and Betulinaldehyde, may play a key role in its anti-inflammatory activity. These findings suggest that EMS has promising potential as a natural anti-inflammatory agent.

The acetic acid-induced writhing test in mice, which models visceral pain, is widely used to evaluate peripherally acting analgesics (Hasan et al., 2010). In this method, pain is induced by triggering a localized inflammatory response, leading to the release of free arachidonic acid from tissue phospholipids via the cyclooxygenase pathway and subsequent prostaglandin biosynthesis (Duarte et al., 1988). Increased prostaglandin levels in the peritoneal cavity enhance inflammatory pain by increasing capillary permeability (Zakaria et al., 2008). Agents that reduce the number of writhing episodes are believed to exert their analgesic effects primarily by inhibiting prostaglandin synthesis, a peripheral mechanism of pain modulation (Ullah et al., 2014). This study highlights the significant analgesic activity of the ethanolic extract of *M. sylvatica*, likely due to bioactive compounds that interfere with prostaglandin synthesis or related pathways. These findings suggest that EMS contains analgesic principles capable of mitigating pain through both peripheral and central mechanisms, offering potential as a natural pain relief alternative. The formalin test, a model of persistent pain involving peripheral inflammation and central sensitization, was used to evaluate the extract's effects. This test induces a biphasic response: an early neurogenic phase caused by direct nerve stimulation and a late inflammatory phase driven by pro-inflammatory mediators like kinins, leukocytes, and prostaglandins (Wheeler-Aceto & Cowan, 1991). Formalin-induced acute inflammation results from cell injury, triggering the release of endogenous pain mediators (Chen et al., 1995). The results demonstrate that EMS produced antinociceptive effects in both phases of the formalin test, indicating its ability to modulate both central and peripheral pain pathways. The analgesic activity of EMS across all nociceptive models suggests that its mechanism of action may involve the arachidonic acid cascade, including the inhibition of lipoxygenase and/or cyclooxygenase pathways, as well as interactions with opioid receptors. In a study, *M. indica* demonstrated a promising analgesic effect in the formalin-induced paw licking test (Garrido et al., 2001). Terpenoids such as 24-Noroleana-3,12-diene, Beta-Carotene, Neophytadiene, and Betulinaldehyde, identified in *M. sylvatica*, are likely responsible for its analgesic properties. These findings underscore the therapeutic potential of EMS as a source of natural analgesic agents.

Virtual screening is a valuable tool that allows researchers to evaluate the theoretical ADME/T (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiles of compounds before their biological activity is experimentally tested (Tareq et al., 2020). This approach enhances the precision of identifying potentially active compounds by focusing on their predicted pharmacokinetic properties, offering an advantage over random screening. In our ADME analysis, all selected compounds adhered to Lipinski's Rule of Five, a key guideline for drug-likeness, which is critical in prioritizing promising drug candidates for further investigation.

Molecular docking analysis provides a systematic approach to predict ligand-protein interactions, offering insights into the biological activity of bioactive compounds. It helps elucidate the possible modes of action and binding interactions between ligands and specific binding sites of target proteins (Emon et al., 2020). To explore the potential biological activities (antioxidant, anti-inflammatory, and analgesic) of *M. sylvatica* (EMS), 24 selected compounds were subjected to docking studies. The targets included human cytochrome P450 CYP2C9 (PDB: 1OG5), human cyclooxygenase-2 (PDB ID: 5IKR), and a cyclooxygenase-2 inhibitor (PDB ID: 6COX). Notably, 24-Noroleana-3,12-diene and γ -Tocopherol exhibited higher binding affinities toward their respective target receptors compared to standard drugs, indicating their potential therapeutic efficacy. The pharmacological and computational evaluations of *M. sylvatica* revealed favorable profiles with very mild toxicity, highlighting its potential as a promising drug candidate. These findings underscore the importance of combining *in silico* approaches with experimental validation to identify bioactive compounds for drug development.

PASS (Prediction of Activity Spectra for Substances) is a computational tool used to predict the biological activities of chemical compounds based on their structural features (Poroikov & Filimonov, 2005). It evaluates the likelihood of a compound exhibiting specific bioactivities by calculating two key parameters: Pa (probability of trustworthy activity) and Pi (probability of trustworthy inactivity). In this study, compounds with a higher Pa value than Pi were considered promising candidates for specific biological activities. The comprehensive analysis revealed significant insights into the selected compounds, suggesting their potential therapeutic effects. These findings may be attributed to the synergistic interactions of various phytochemicals present in the extract, including both well-established and previously unreported phytochemicals. This highlights the importance of exploring the combined contributions of multiple bioactive components in natural products for the discovery and development of new drugs.

This study successfully demonstrates the significant antioxidant, anti-inflammatory, analgesic, and acute toxicity profiles of *M. sylvatica* leaves extract using well-established *in vitro* and *in vivo* assays. However, specific advanced assessments, such as *in vivo* anti-inflammatory model (e.g., carrageenan-induced edema, formalin-induced paw edema), enzyme-based inflammatory biomarker assays (e.g., COX-2, IL-6), and *ex vivo* evaluation of inflammatory mediators, including prostaglandins, leukotrienes, oxyradicals, and nitric oxide, were not conducted in this investigation. While these analyses would provide deeper mechanistic insights into the anti-inflammatory pathways, the current tests employed have adequately reflected the extract's pharmacological potential through observable biological effects. Furthermore, our toxicity assessment was confined to acute toxicity measures, including hematological, and histopathological investigations. Sub-acute (28-day) and chronic (90-day) toxicity tests, in accordance with OECD requirements, were not performed. Subsequent research should incorporate *in vivo* efficacy models and prolonged toxicity investigations to confirm therapeutic significance, ascertain long-term safety, and facilitate prospective clinical translation. The results presented here form a strong foundation for future studies aiming to explore the precise molecular and biochemical mechanisms. The limitations noted do not diminish the significance of the findings but rather highlight areas for further research to expand understanding.

5 | Conclusion

The findings of this study highlight the therapeutic potential of *Mangifera sylvatica* leaves as a promising source of natural agents for managing pain and inflammation. The ethanolic extract exhibited notable analgesic and anti-inflammatory effects, which may complement existing conventional therapies. Additionally, its antioxidant activity, as demonstrated through DPPH scavenging and reducing power assays, supports the presence of bioactive constituents capable of mitigating oxidative stress. GC-MS/MS analysis confirmed a diverse phytochemical profile, particularly enriched with phenolic compounds. *In silico* molecular docking further identified 24-Noroleana-3,12-diene and γ -Tocopherol as lead candidates due to their strong binding affinities, suggesting significant pharmacological relevance. Taken together, these results provide a solid foundation for future investigations into the bioactive components of *M. sylvatica*, with the intention of developing novel plant-based therapeutics and elucidating their mechanisms of action.

Declarations

Conflict of Interest

The authors declare that they have no competing interests.

Data Availability Statement

Data will be available upon request.

Authors' Contribution

Imran Hossain: Project Design, Conceptualization, Plant Collection, Methodology, Investigation, Data curation, data analysis, writing, review & editing; **Mohammad Minhazul Abedin:** Plant Collection, Investigation, Data curation, data analysis, review & editing; **Mahathir Mohammad:** Methodology, Investigation, Data curation, data analysis, writing, review & editing; **Fariha Tasnim:** Investigation, Methodology, Data curation, data analysis, writing; **Fahmida Tasnim Richi:** Methodology, Investigation, Data curation, data analysis, review & editing; **Mohammed Aktar Sayeed:** Supervision, methodology, conceptualization, review & editing; **Md. Hossain Rasel:** Investigation, Data curation, data analysis, Software, writing; **Md. Jahirul Islam Mamun:** Investigation, Data curation, data analysis, writing; **Sayed Al Hossain Rabbi:** Data analysis, Data curation, writing. **Safaet Alam:** Supervision, Methodology, Investigation, Data curation, data analysis, review & editing,

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Table

Table 1 is available in the Supplementary Files section.

Figures

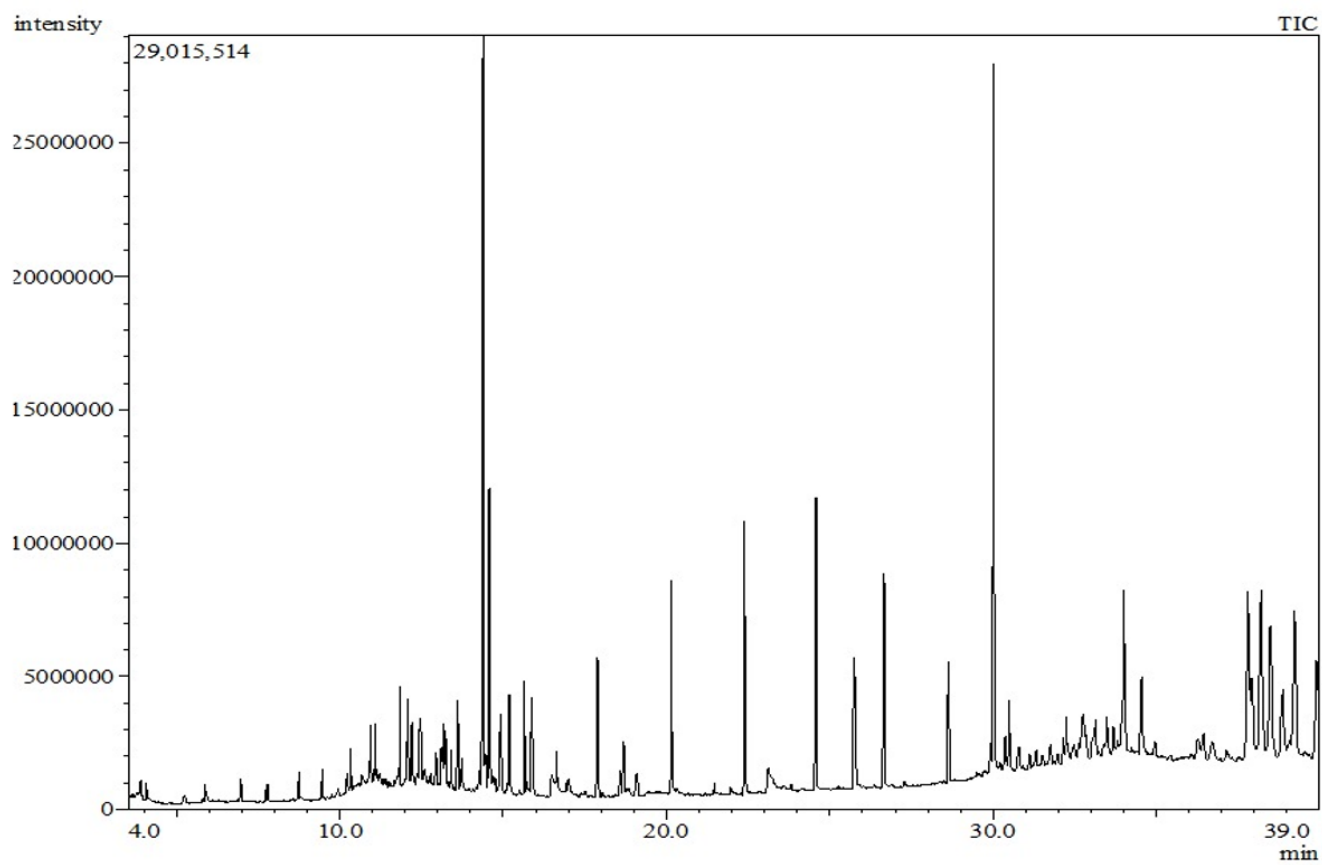
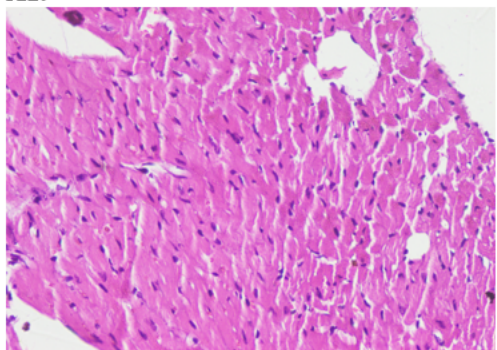


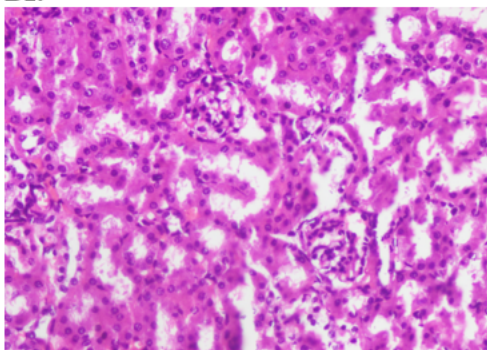
Figure 1

GC-MS/MS Chromatogram of the Ethanolic Extract of *M. sylvatica*.

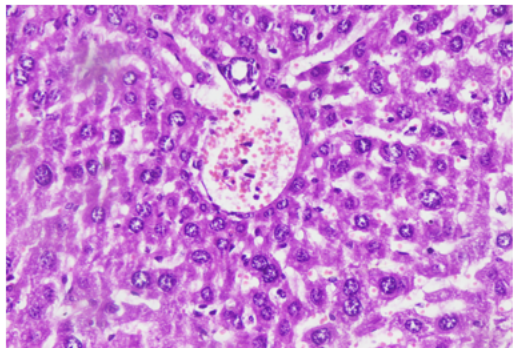
A1.



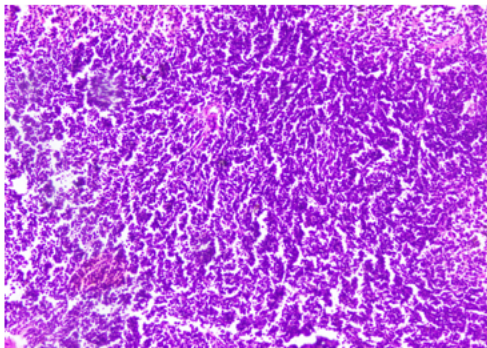
B1.



C1.



D1.



E1.

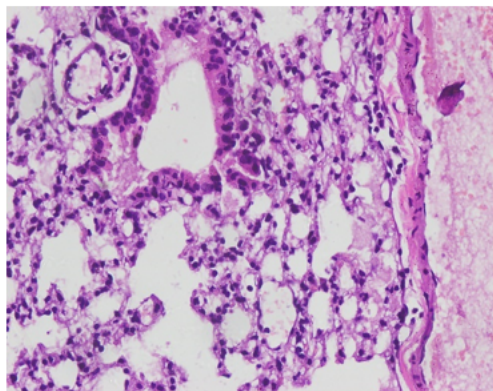


Figure 2

Histopathology observation of Acute Toxicity of EMS 4000 mg/Kg body weight mice at 400x magnification. **A1.** Heart **B1.** Kidney **C1.** Liver **D1.** Spleen **E1.** Lungs. EMS= Ethanolic Extract of *M. sylvatica*.

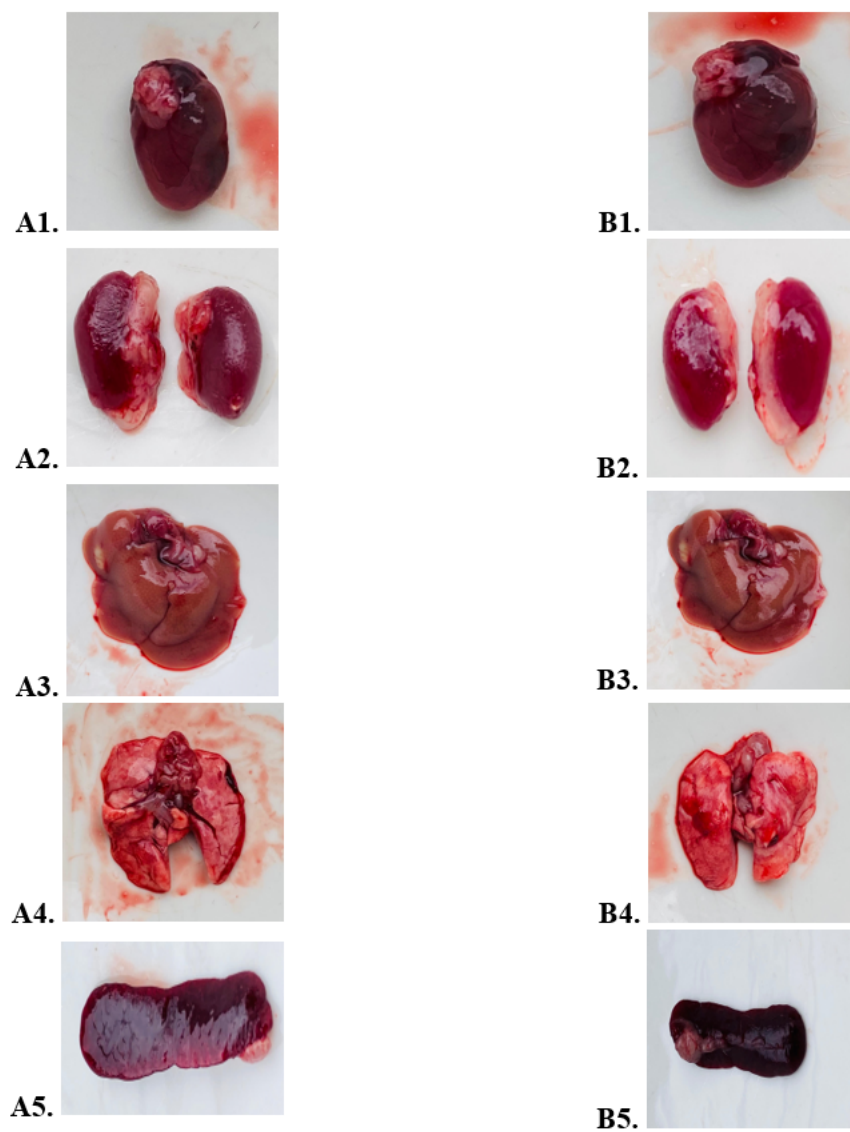


Figure 3

The gross observations of systemic organs from EMS Control (A) and EMS 4000 mg/ Kg body weight treated(B) mice are shown: Heart (**A1** and **B1**), kidney (**A2** and **B2**), Spleen (**A3** and **B3**), lung (**A4** and **B4**), and liver (**A5** and **B5**)

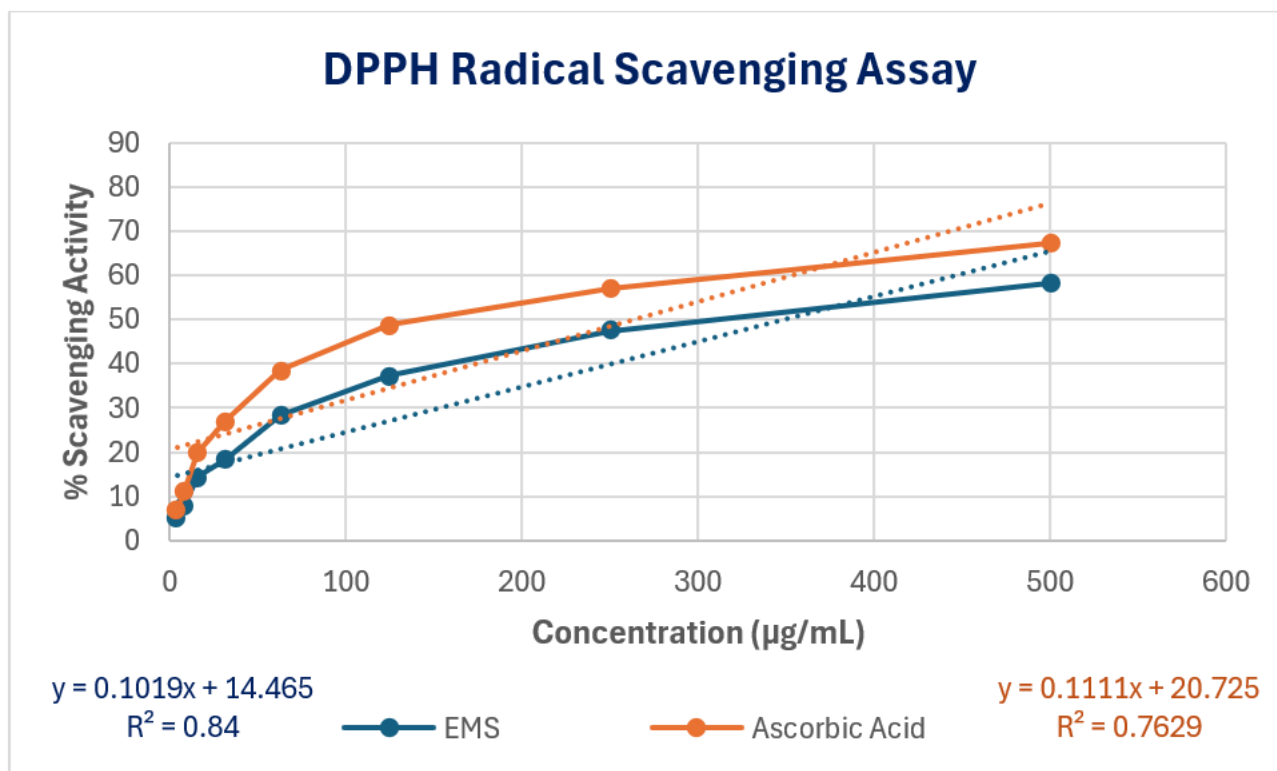


Figure 4

Antioxidant Activity of EMS and the Standard Ascorbic Acid. EMS= Ethanolic Extract of *M. sylvatica*.

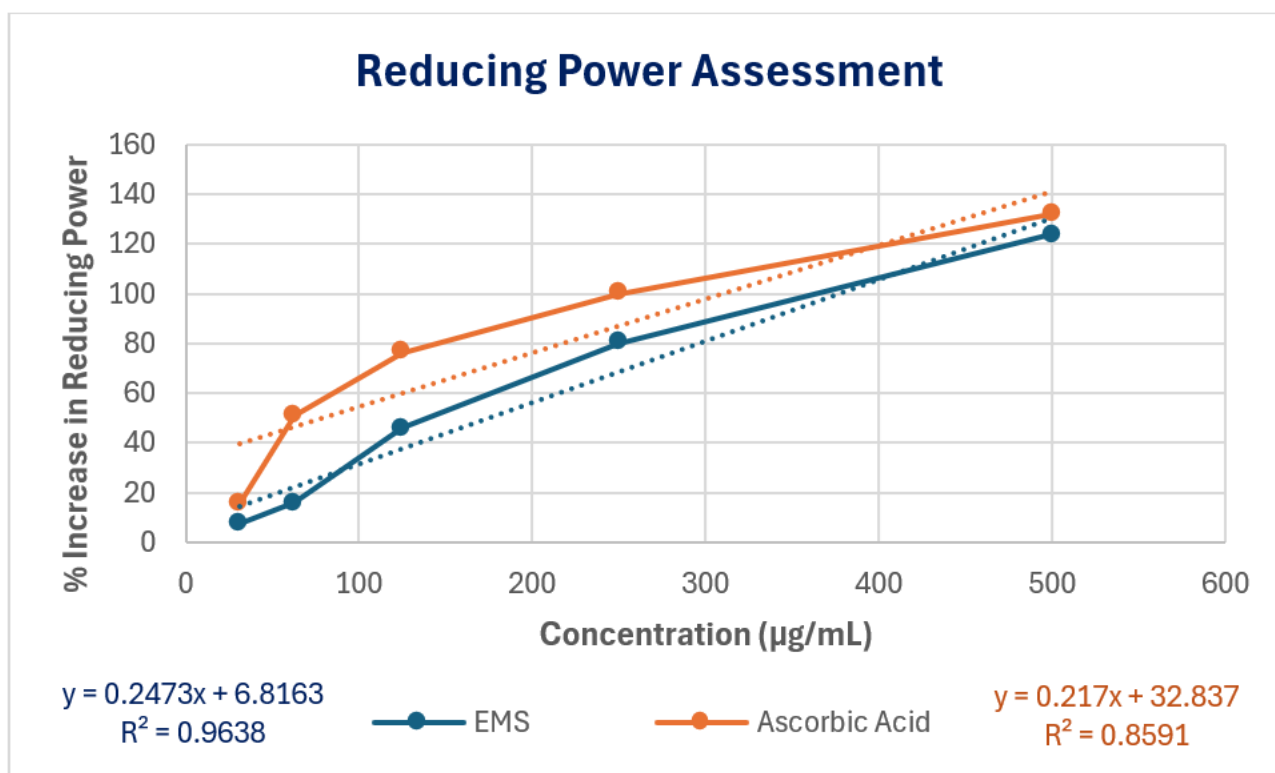


Figure 5

Values are Mean $\pm$ SEM, Reducing Power of EMS, and Ascorbic Acid by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation. EMS= Ethanolic Extract of *M. sylvatica*.

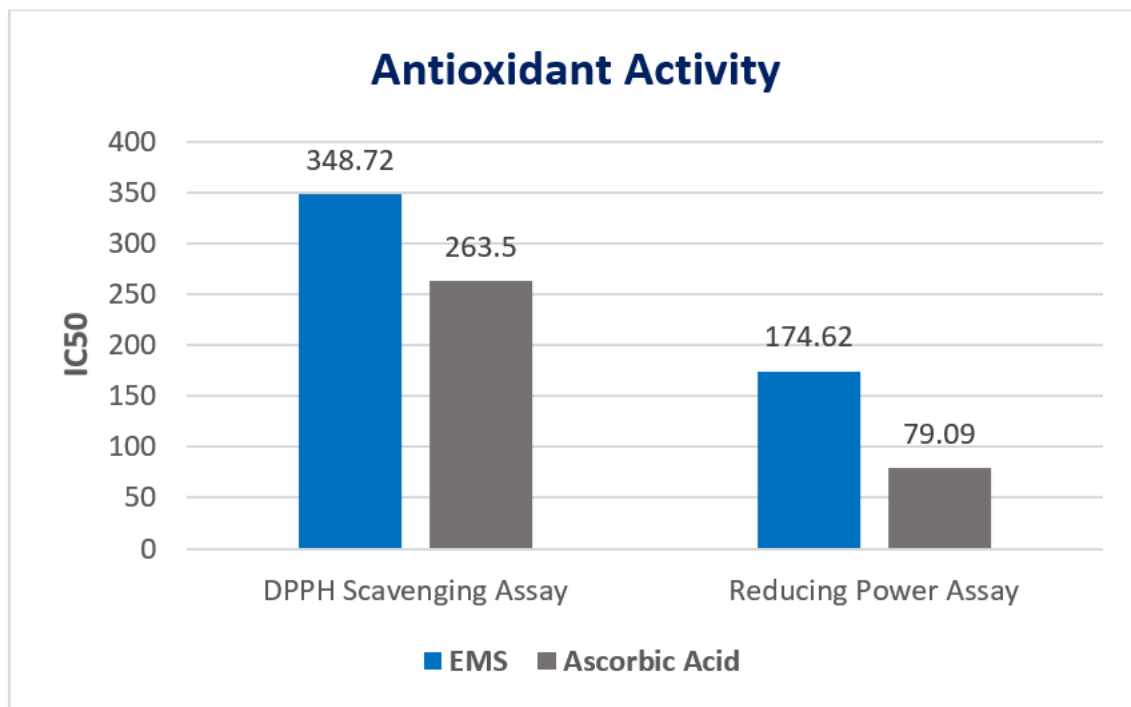


Figure 6

IC₅₀ Comparison of EMS and the Standard Ascorbic acid in DPPH Scavenging and Reducing Power Assay.

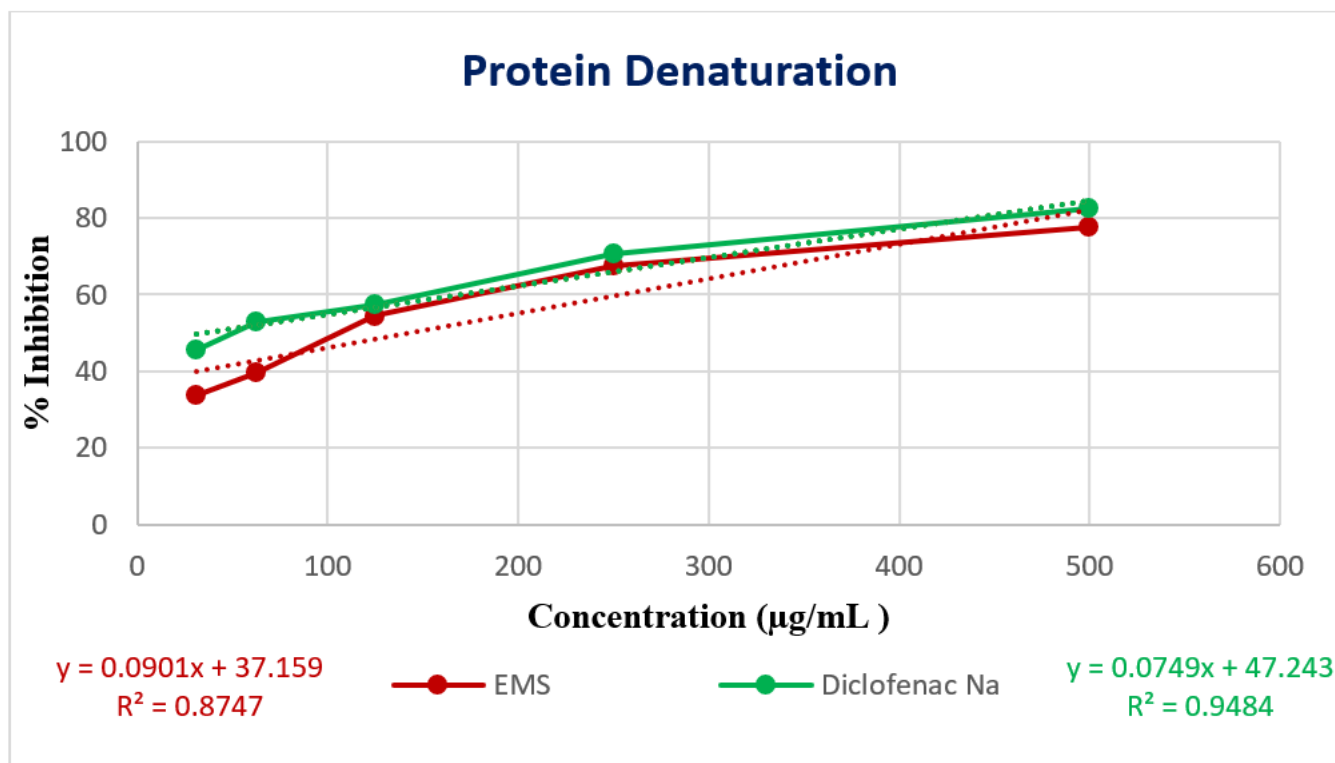


Figure 7

Anti-inflammatory Activity of EMS and the Standard Diclofenac Na. EMS= Ethanolic Extract of *M. sylvatica*.

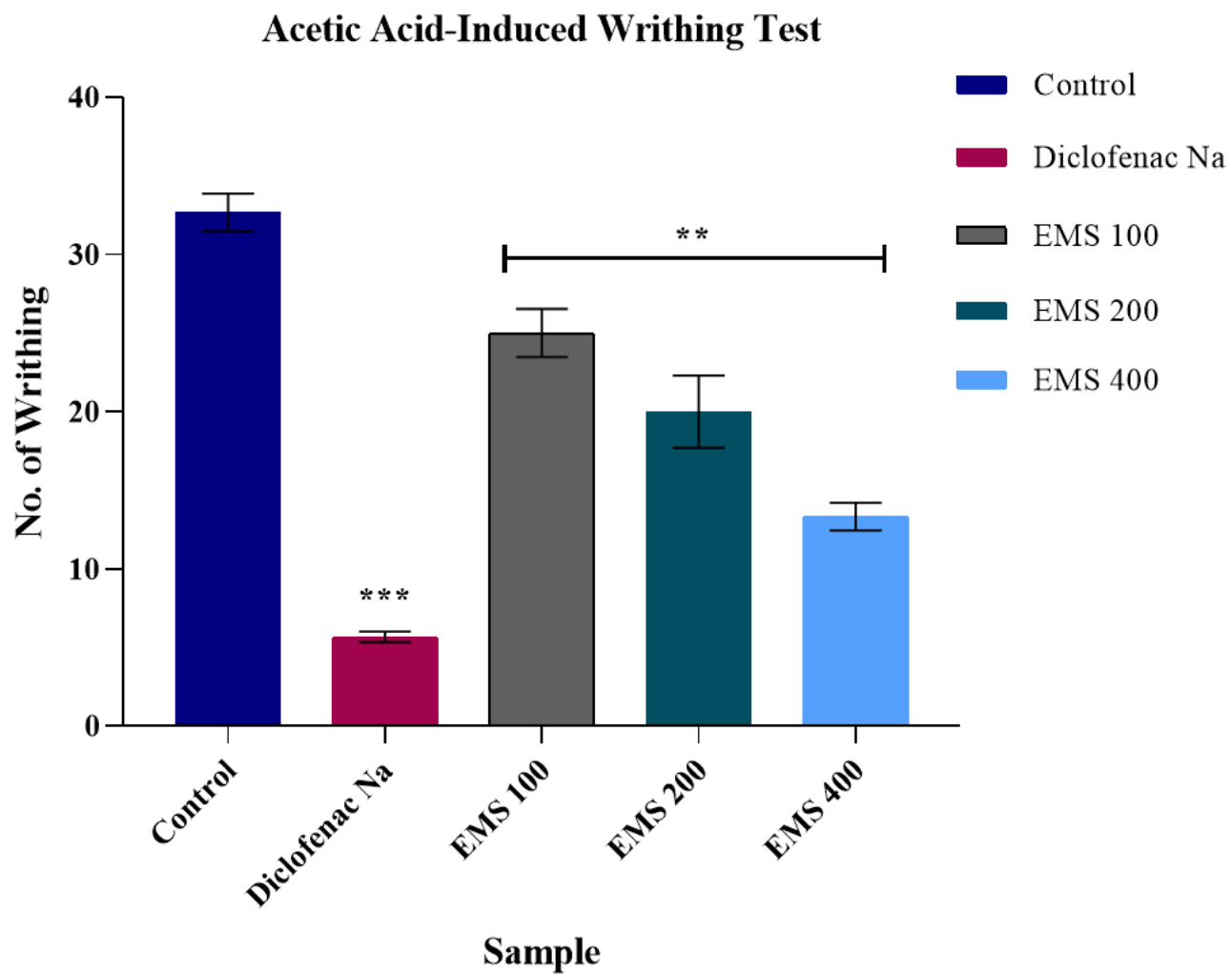


Figure 8

Assessment of Analgesic Activity of EMS through Acetic Acid-Induced Writhing Test. EMS= Ethanolic Extract of *M. sylvatica*.

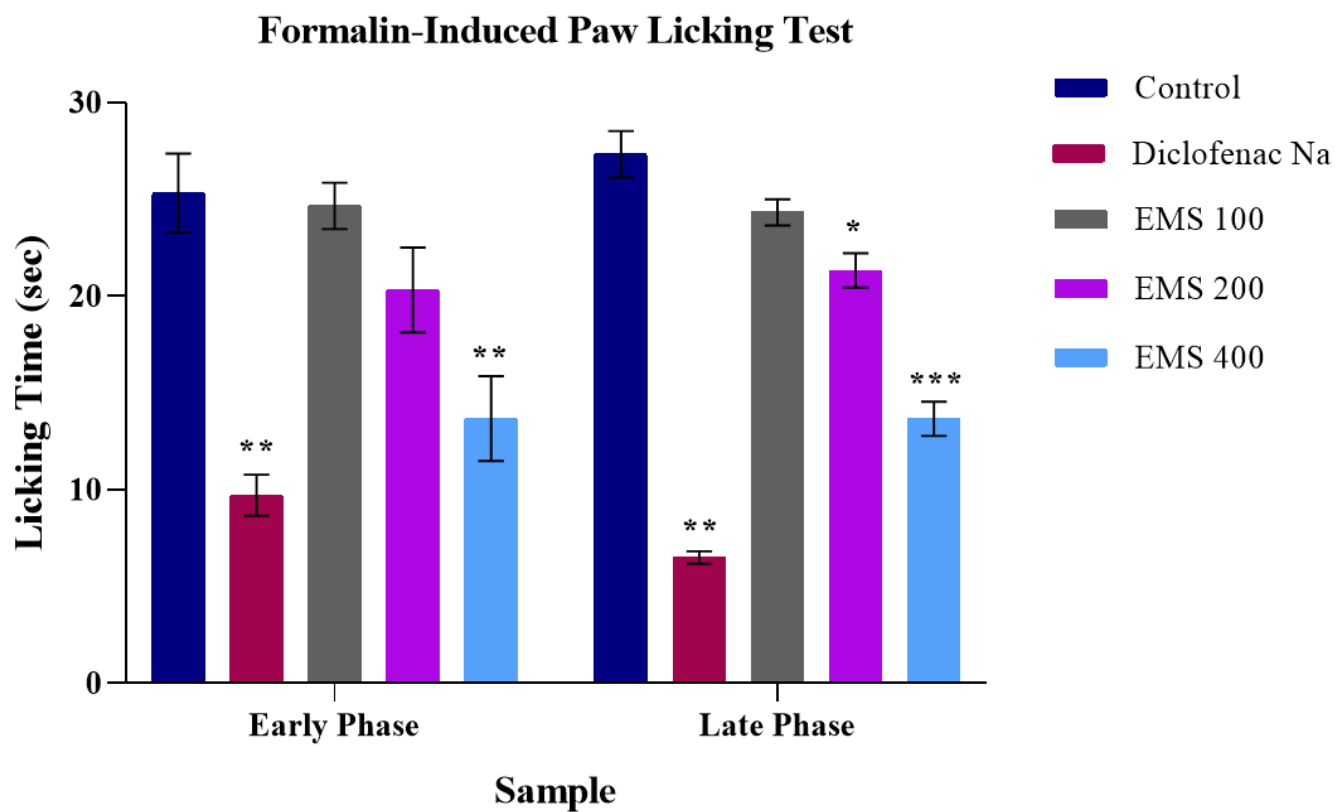
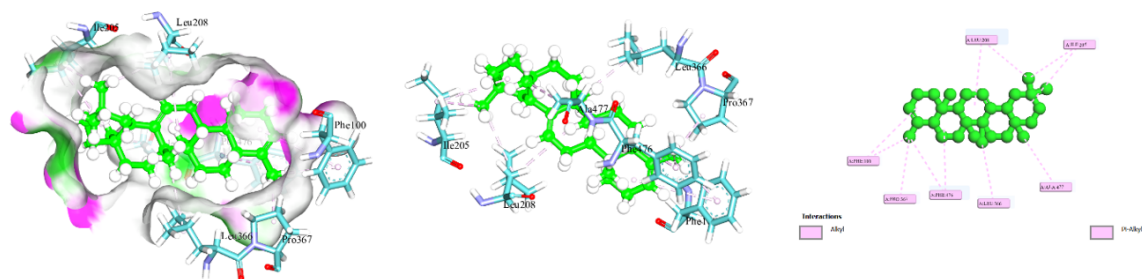


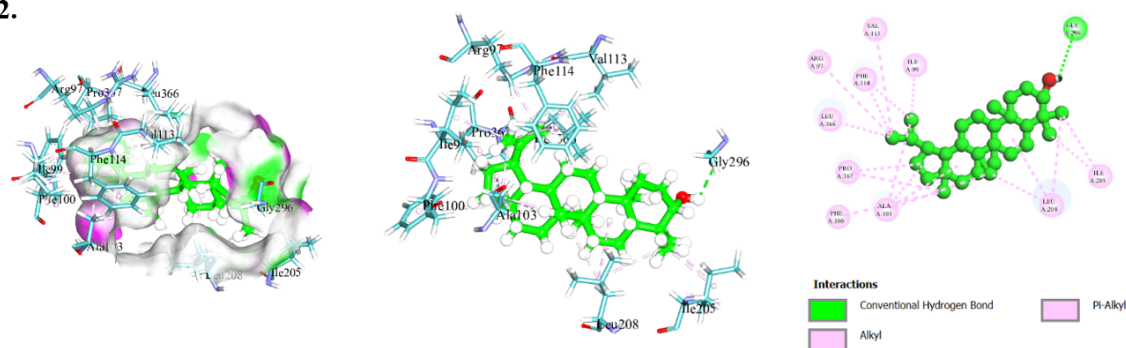
Figure 9

Assessment of Analgesic Activity of EMS through Formalin-induced Paw Licking Test. EMS= Ethanolic Extract of *M. sylvatica*.

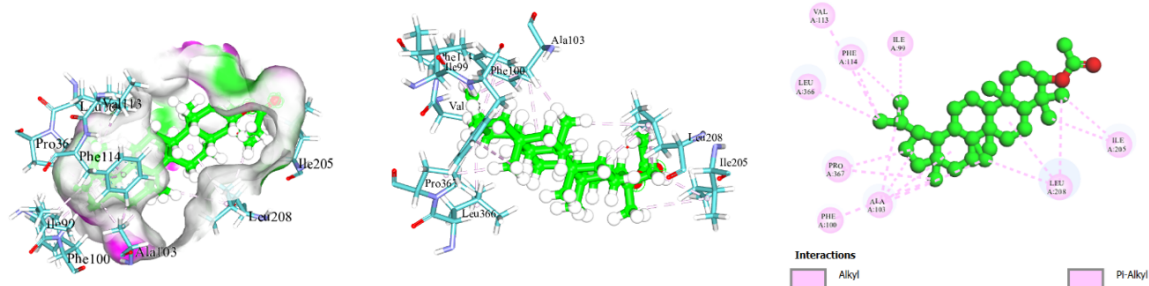
A1.



A2.



A3.



A4.

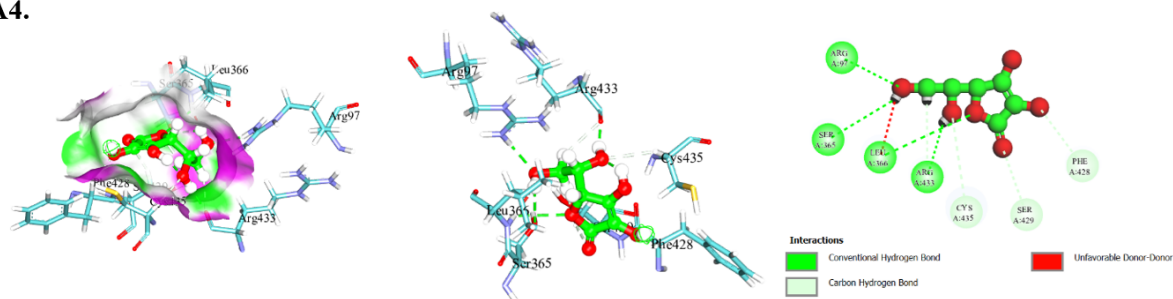
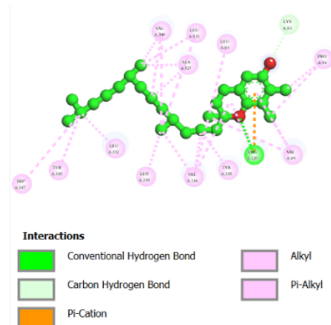
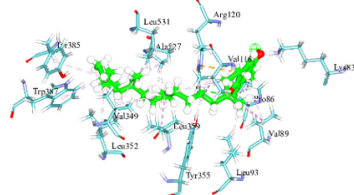
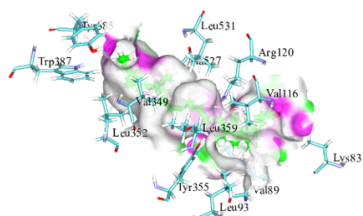


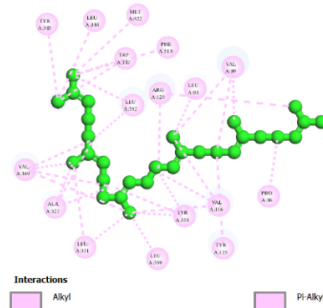
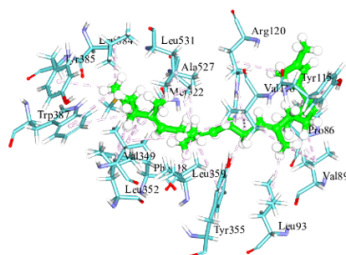
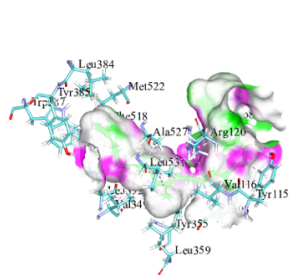
Figure 10

Molecular docking interaction of compounds against the human cytochrome P450 CYP2C9 (PDB: 1OG5), where A1. 24-Noroleana-3,12-diene A2. Lupeol A3. Lup-20(29)-en-3-ol, acetate A4. Ascorbic acid (Standard).

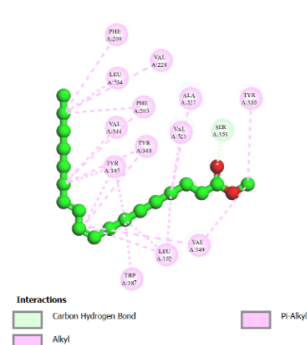
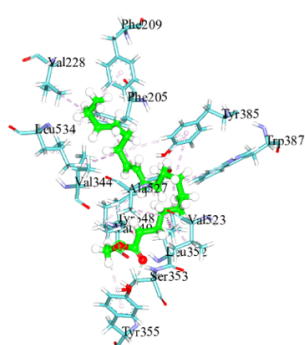
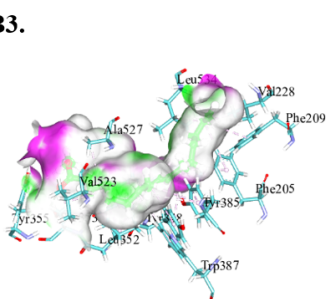
B1.



B2.



B3.



B4.

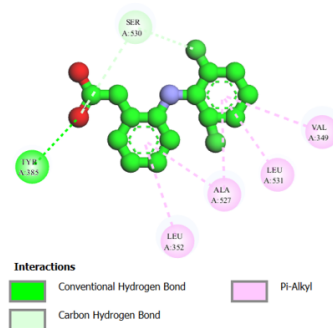
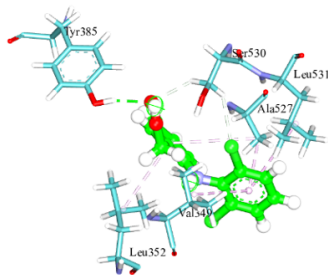
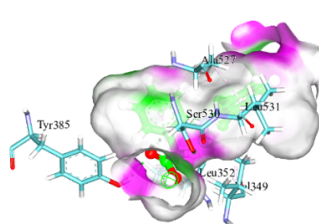
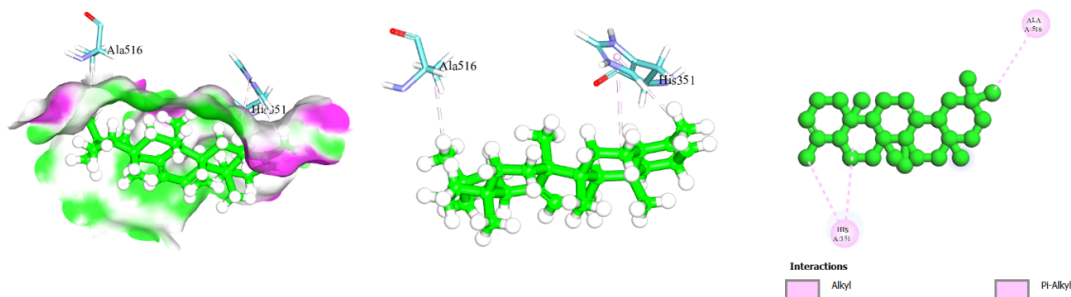


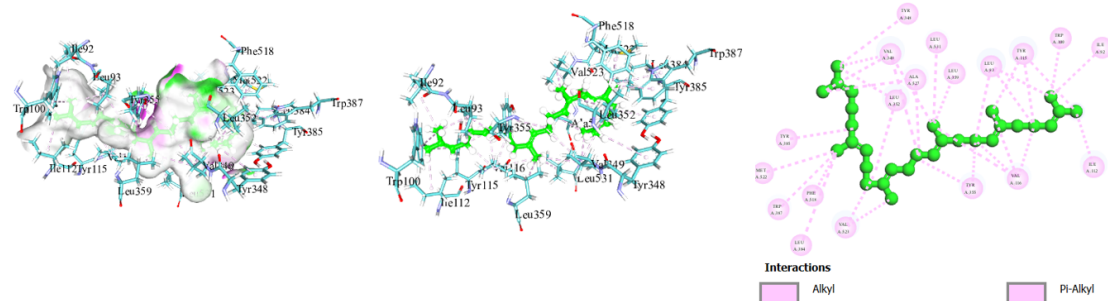
Figure 11

Molecular docking interaction of compounds against the human cyclooxygenase-2 (PDB ID: 5IKR), where B1. γ -Tocopherol B2. Squalene B3. Methyl 9,11-octadecadienoate B4. Diclofenac Na (Standard).

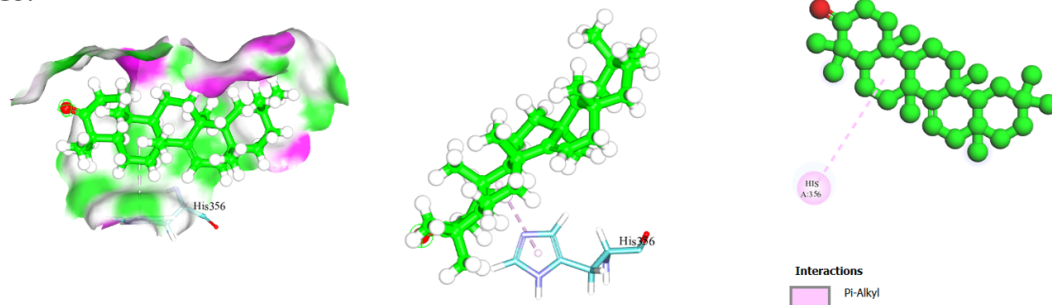
C1.



C2.



C3.



C4.

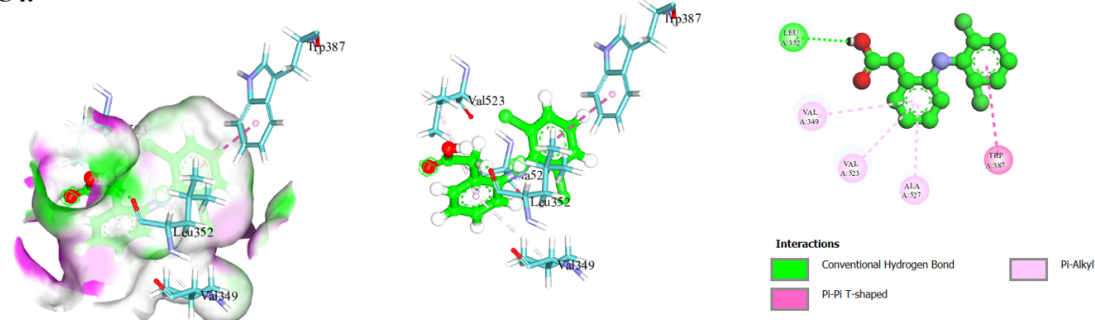


Figure 12

Molecular docking interaction of compounds against the cyclooxygenase-2 inhibitor (PDB ID: 6COX), where C1. 24-Noroleana-3,12-diene C2. Squalene C3. Friedoolean-14-en-3-one C4. Diclofenac Na (Standard).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx