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ORIGINAL RESEARCH

Anti-Inflammatory, Analgesic and Anti-Oxidant Effects of *Shirakiopsis Indica* (Willd). Fruit Extract: A Mangrove Species in the Field of Inflammation Research

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Background: *Shirakiopsis indica* (Willd)., commonly known as Sa-Mor-Ta-Lay in Thailand, is a mangrove plant belonging to the Euphorbiaceae family. As mangrove plants' medicinal potentials are less explored, this study sought to qualitatively and quantitatively verify the bioactive components of *Shirakiopsis indica* fruits methanolic extract (SIF-ME) at the side of its analgesic, anti-inflammatory and anti-oxidant effects followed by in-silico studies.

Methods: The in-vivo assessments of analgesic activity involved the hot plate test, acetic acid-induced writhing test, and formalin-induced licking test. The anti-inflammatory efficacy was assessed through the human RBC membrane stabilization assay (HRBC), protein denaturation assay, and xylene-induced ear edema methods. Antioxidant potential was implemented by the DPPH scavenging method.

Results: The SIF-ME consistently displayed significant anti-nociceptive activity in a dose-dependent pattern ($p < 0.05$). The maximum analgesic activity was found in the highest dose (200 mg/kg; $p < 0.001$) in a hot plate, acetic acid-induced writhing test 43.47%, and in formalin-induced licking test in both early phase (43.3%; $p < 0.01$) and late phase (61.84%; $p < 0.001$%). The extract provided optimal protection against hemolysis (83.41% decrease) at 1000 µg/mL and significantly inhibited protein denaturation (67.34–26.05%) at doses of 1000–62.5 µg/mL. At 200 mg/kg, the extract showed dose-dependent and substantial inhibition (54.07%; $p < 0.01$) of xylene-induced ear edema. The in-vitro DPPH (IC_{50} = 469.5 µg/mL) results showed remarkable scavenging activity and concentration-dependent reducing power. The extract demonstrates no acute oral toxicity, as indicated by an LD_{50} value exceeding 1000 mg/kg body weight. Gas Chromatography-Mass Spectrometry/Mass Spectrometry (GC-MS/MS) analysis was performed which yielded sixty bioactive compounds. In-silico and molecular docking studies revealed favorable pharmacological properties, including good binding affinities and ADME/T profiles.

Conclusion: These results support the medicinal use of the plant, which makes it a potential source of analgesic, anti-inflammatory, and antioxidant candidates.

Keywords: natural products, medicinal plants, *shirakiopsis indica*, acute oral toxicity, analgesic, anti-inflammatory, antioxidant, GC-MS/MS, molecular docking

Introduction

Pain is an undesirable warning signal in the nervous system, a response to possible tissue destruction (the taxonomy of pain). It occurs through painful stimuli and is transmitted through specific neural pathways, representing an intrinsic

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pathway.[1] A considerable increase is noticed in the number of patients choosing complementary and alternative therapy and taking plant extracts from folkloric medicine. Along with the mode of action being more significant than that of NSAIDs and analgesics, herbal medicinal items have reduced adverse effects.[2] Steroidal drugs, non-steroidal drugs, and immune-suppressive drugs are consistently utilized by individuals worldwide to alleviate inflammatory conditions. Through the process of suppressing cyclooxygenase (COX) enzymes.[3] Nevertheless, this medication frequently exhibited a correlation with significant detrimental outcomes, including gastrointestinal hemorrhaging and peptic ulceration.[4] Moreover, they impact the kidneys, liver, and cardiovascular system.[5] NSAIDs are harmful to pathways, inducing ROS injury, and antioxidants are a natural treatment for mitigating toxic effects.[6] Without proper treatment and management, chronic illnesses can lead to significant disabilities and have the potential to be lethal.[7] Oxidative stress and inflammation are closely intertwined processes implicated in various chronic diseases and leading to cellular damage and potentially chronic inflammation.[8] Reactive oxygen species (ROS) generated by cells, such as endothelial, inflammatory, and immunological cells, have a dual purpose: they are involved in redox signaling and also contribute to oxidative stress or damage. The ethanolic extract of *Shirakiopsis indica* exhibited the highest inhibition of nitric oxide production as an anti-oxidant effect.[9] Oxidative stress and inflammation can lead to cellular damage. Degenerative disease, cancer, cardiovascular disease, and metabolic syndrome.[10] Flavonoids in the chloroplast can help maintain the envelope membrane's integrity by altering lipids during cellular dehydration, therefore protecting against oxidative damage.[11] Plants possess sophisticated defensive mechanisms against reactive oxygen species (ROS).[12] Plant tissues contain several phenolic chemicals such as flavonoids, tannins, and lignin precursors that can act as antioxidants by scavenging ROS, in addition to the tocopherols.[13]

Natural products (NPs) refer to a vast array of diverse chemical compounds that exhibit a broad range of biological functions. These NPs have been extensively utilized in several fields, including human and veterinary medicine, as well as agriculture.[14] Plants remain a primary probable resource for new medications and chemicals. Due to the availability, affordability, and inefficiency of conventional drugs, herbal medications are being used extensively for healing diverse disease conditions in many regions of the world.[15] Medicinal herbs have served as a resource for a wide range of biologically active chemicals, most of which have possible applications in drug development, and they continue to be the source of lead compounds.[16]

Shirakiopsis indica (Willd). is found in East Asia and belongs to the Euphorbiaceae family and *Shirakiopsis* genus. Its presence is widespread near water bodies like rivers, seashores, and mangrove forests, thriving in various forest types, wetlands, and elevations up to 75 meters. Thai traditional medicine has utilized a variety of herbs to address gastritis and peptic ulcers.[17,18] The predominant plant utilized for managing gastrointestinal issues is the fruit of *Shirakiopsis indica*, known as Sa-Mor-Ta-Lay in Thai.[9] The seeds are rich in drying oil, contain a drying oil that is edible when mature and are used as vegetables or condiments, but the unripe fruit wall contains latex, which produces blisters on the skin and poison for fish. It has long been used to cure digestive disorders, gastric inflammation, etc. The ethanolic extract of *Shirakiopsis indica* had the most potent cytotoxic effects on the Kato III gastric cancer cell line and the most significant suppression of nitric oxide generation, as demonstrated by the previously reported optimal IC_{50} values.[9] The most prevalent non-erosive gastritis identified by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) is classified as non-erosive gastritis and is caused by the grade I carcinogenic bacteria *H. pylori*, and it is mainly prevented by this plant[19] Aesculetin, a compound isolated from fruits, is classified as a 6.7-dihydroxy coumarin, a subclass of the broader coumarin family of organic compounds. This study investigates the analgesic, anti-inflammatory, and antioxidant properties of *Shirakiopsis indica* fruit methanolic extract (SIF-ME) from Bangladesh, as its pharmacological effects have not been previously reported.

Materials and Methods

Plant Collection

Dried fruits of *Shirakiopsis indica* (Willd). were collected from the rural area of Anowara in Chattogram, Bangladesh in July 2022. The samples were taxonomically verified by Professor Dr. Shaikh Bokhtear Uddin from the University of Chittagong, Bangladesh. A voucher specimen was assigned the accession number (3632) and deposited in the herbarium center of the University of Science and Technology, Chittagong.

Extraction Methods

Collected fruits were cleaned and shade-dried at around 25°C for 4 weeks to prepare them for further processing. Dried *Shirakiopsis indica* fruits (2 kg) were undersized within a mortar and powdered using a blender. Then 500 grams of powder were macerated with 2 liters of 95% methanol for 3 days. This macerated process was repeated two times more with the crude residue. The extract was filtered through Cotton and Whatman filter paper. The filtrate was dried by a rotary evaporator (RE200, Bibby Sterling, UK). Until the experiment was conducted, SIF-ME was stored in a glass vial at 4 °C.[18]

Chemicals

Methanol, acetic acid, chloroform, and formalin were obtained from a local scientific store. Diclofenac sodium was received from General Pharmaceuticals Ltd, Bangladesh. DPPH, Folin-ciocalteu reagent, and other solvents are provided by the University of Science and Technology Chittagong. All other chemicals used in this experiment were of analytical grade.

Yield Percentage

The yield percentage (%) of the extract was determined as the following equation:

$$\text{Yield}\,(\%) = \frac{Dry\ extract\ weight}{Dry\ powder\ weight} \times 100$$

Experimental Animals and Ethical Statement

Swiss albino mice, weighing 22 to 30 g, were obtained from the animal research division of Comilla University, Comilla, Bangladesh. The Animals were kept in poly-carbonated cages under standard conditions (25 ± 2°C, 55–60% humidity) with a 12-hour/daylight cycle.[20] The animals involved in this study had adequate access to water and food. The protocols for this research were approved by the Institutional Animal Ethics Committee of the Department of Pharmacy at the University of Science and Technology Chittagong, Bangladesh (approval number USTMEBBC/23/07/23).

Qualitative Analysis

The freshly produced SIF-ME underwent a qualitative examination to detect the presence of various chemical components. The analysis included tests for the following compounds: alkaloids, carbohydrates, flavonoids, glycosides, tannins, steroids, gums, and saponins.[21]

Quantitative Analysis

Total Phenolic Content (TPC)

The TPC of SIF-ME was determined using Folin-Ciocalteu reagent (FCR) as the oxidizing agent. After being decreased by FCR via this technique, the test sample's polyphenolic levels take on a blue hue. Twenty percent (w/v) Na_2CO_3 (2mL) and 200 µg/mL extract were combined with 0.5 mL of FCR that had been diluted in 3 mL of distilled water. The solution was incubated in the dark at 25°C for 60 minutes. The absorbance was measured at 650 nm using the UV spectrophotometer. The absorbance was taken two times more. A standard curve was created using gallic acid to calculate the TPC, and the TPC results were expressed in milligrams of gallic acid equivalents (GAE) per gram. Consequently, the same strategy for FCR reduction was utilized: plotting concentration VS absorbance allowed for creating a standard calibration curve for a known amount of gallic acid.[22]

$$Y = 0.0019x + 0.0135; R^2 = 0.9984$$

Where Y is the absorbance and x is the gallic acid equivalent (mg/g).

Total Flavonoid Content (TFC)

Utilizing the aluminum chloride technique, the total flavonoid content of the extract was assessed. In short, 0.3 mL of a 5% $NaNO_2$ solution was combined with 50 µL of crude extract (1 mg/mL) that had been diluted to 1 mL with methanol and 4 mL of distilled water. Following a 5-minute incubation period, 0.3 mL of a 10% $AlCl_3$ solution was added. The combination was then allowed to rest for 6 minutes before 2 mL of 1M NaOH was added and made up to 10 mL with

double-distilled water. At room temperature, the combination was given a 15-minute rest. Using distilled water as a blank control. The absorbance was measured at 510 nm.[23] Each analysis was done three times. Based on the calibration curve, the flavonoid content was determined by calculating the quercetin concentration (mg/g) using the following equation:

$$Y = 0.0013x + 0.0451; R^2 = 0.9971$$

Y is the absorbance and x is the quercetin equivalent (mg/g)

Total Tannin Content (TTC)

The tannins were determined using the Folin-Ciocalteu reagent (FCR), as reported by Amorim et al.[24] Briefly, 50 µL of the sample extract is added with 950 µL of distilled water and 500 µL of FCR. 2.5 mL of a 35% Na_2CO_3 solution and dilute to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 40 minutes, and absorbance was measured at 725 nm. A blank was prepared with distilled water instead of a sample. The tannin results were determined as mg of tannic acid equivalent per gram of extract using the equation obtained from a standard tannic acid calibration curve.

$$Y = 0.21x + 0.0135; R^2 = 0.9998$$

Where Y is the absorbance and x is the Tannic acid equivalent (mg/g).

GC-MS/MS Analysis

Utilizing the electron impact ionization (EI) approach, the advantageous compounds extracted from SIF-ME were analyzed by the use of a mass spectrometer (GC–MS/MS TQ 8040, Shimadzu, Kyoto, Japan) connected to a gas chromatograph (GC–MS/MS, Shimadzu, Japan). Utilized as a fused silica capillary column with the following requirements: Rxi-30 m, 0.25 mm ID, 0.25 m, and 5 m. The column's temperature was adjusted to 50 °C. Split mode was used to deliver each specimen, and a constant injection temperature of 250 °C was used. The oven was warmed for one minute at 500 °C, two minutes at 200 °C, and seven minutes at 300 °C. The gas chromatography system operated at a pressure of 53.5 kPa, with a total flow rate of 11.0 mL/min and a column flow rate of 1.0 mL/min. The chemical names, structures, and molecular masses of the bioactive components in each extract were determined by comparing the mass spectra of each extract with the data included in the NIST and Wiley libraries. The whole run time needed to complete the GC-MS /MS analysis was 39 minutes. The detector's voltage (relative mode) was 0.6 kV. The contact was 250 °C in temperature, while the ion source was 230 °C. The solvent cut time was 3.5 minutes. With a m/z value of 50 to 600, the Q3 scan mode was used for data gathering. In qualitative GC-MS/MS analysis, relative abundance compares each compound's peak area to the total or most abundant peak, allowing identification based on fragmentation patterns and relative quantities.[25]

Acute Oral Toxicity

The OECD criteria and defined protocol were adhered to in the performance of the acute oral toxicity test.[26] A single oral dosage of the test extract (SIF-ME) at 100, 200, 400, 500, 1000, and 2000 mg/kg of body weight was given to the assigned animals (n = 5). The mice were fasted for the whole night before the extract was given. Food was not allowed for a further 3–4 hours after administration. Individual experimental animals were observed for the first half hour following dosage, then at regular intervals for the next 14 hours, all while being closely watched for any odd reactions, such as behavioral abnormalities, allergic reactions (itching, swelling, skin, and rash), and death.[27]

Evaluation of Anti-Nociceptive Activity

Hot Plate Method

After placing on Eddy's hot plate, mice were observed at 0, 30, 60, and 120 min, with 55 ± 0.5 °C and 30s a cut-off time. The treatment groups included two dosages (100 and 200 mg/kg body weight), diclofenac sodium (10 mg/kg) as a positive control, and vehicle (10 mL/kg) as a negative control. Nociceptive responses were recorded (licking, flicking or jumping).[28] Percentage of maximum analgesia (% PMA), was calculated.

$$\%\,PMA = \frac{Latency\ test - Latency\ pre\ drug\ drug}{Cut\ of\ time - Latency\ pre\ drug} \times 100$$

Acetic Acid-Induced Writhing Test

This technique involves giving the experimental animal intraperitoneal acetic acid to induce pain. The animals were divided into 4 groups: Control (1% Tween 80), standard group (Diclofenac sodium 10 mg/kg), and two treatment groups (100 and 200 mg/kg of SIF-ME) were given, following an overnight fast. Test samples and vehicle were orally administered 30 minutes before intra-peritoneal delivery of 0.7% v/v acetic acid solution. For observation, each animal was housed individually under a glass container. Every group was watched one by one for how many writhes they produced in ten minutes, starting as soon as the acetic acid solution was intra-peritoneally administered. Not all writhing was performed by the animal fully, as occasionally the animals would begin to writhe but stop short of finishing the movement. This was regarded as half-writhing since it was unfinished. As a result, two partial writhings were interpreted as a single total writhing. The writhing count was compared between a control group and the experimental group.[29] Inhibition (%) was calculated by using a specific formula:

$$Writhing\ Inhibition\,(\%) = \frac{Control\ writhing - test\ writhing}{Control\ writhing} \times 100$$

Formalin-Induced Licking Test

The experimental setup for this study was described in detail in the previous work.[30] The mice were divided into 4 groups: Group-1: Control (1% Tween 80.1 mL/kg, i.p)., Group-2: standard (diclofenac sodium,10 mg/kg), and Groups- 3 and 4: treated groups (SIF-ME 100 and 200 mg/kg body weight). 30 minutes after this treatment, each mouse's left hind paw's plantar area was subcutaneously injected with 50 µL of a newly made 0.6% formalin solution. For 1 hour, each mouse was kept under observation. Paw Licking and biting time (sec) measured as an indicator of discomfort reaction. Two stages were used to determine the anti-nociceptive impact. After the formalin injection, the first (0–5) minutes were recorded as the early phase (phase-1) and the latter (20–30) minutes as the late phase (phase-2).[31] The inhibition (%) was calculated by using a specific formula:

$$Inhibition\,(\%)\ of\ licking = \frac{Total\ number\ of\ licking\,(control\ -\ test\ group)}{Control} \times 100$$

Evaluation of Anti-Inflammatory Activity

Anti-Inflammatory Activity

Human RBC (HRBC) Membrane Stabilization Assay

Cell-Wash and Solvent Preparation. A healthy human volunteer donated blood because they had not consumed any NSAIDs during the two weeks leading up to the trial. Alsever's solution (glucose 2g, Na-citrate 0.8 g, citric acid 0.5 g, NaCl 0.42 g + up to 100 mL DW) was used as an anticoagulant with an equivalent volume of blood. The supernatant was removed by centrifuging for 5 minutes at a speed of 3000 revolutions per minute (rpm). The iso-saline solution of 10 mL (2.7 g NaCl + up to 300 mL DW) was used for washing. The supernatant was cleaned thrice, and packed cell volume was measured. To reconstitute cellular components, phosphate-buffered (10 mM, pH 7.4) was constituted in 200 mL of distilled water: NaH_2PO_4-3.2 g; Na_2HPO_4-1.6 g.[32]

Hypotonicity-Induced Human Red Blood Cell Hemolysis. The anti-inflammatory effect of human red blood cells was evaluated by stabilizing their membrane. The test combination included 1mL extract at various concentrations of 62.5, 125, 250, 500, and 1000 µg/mL, along with 1 mL of phosphate buffer, 2 mL of hyposaline, and 0.5 mL of human RBC suspension. Diclofenac sodium was used as the reference medication, and 2 mL of distilled water served as the control. After incubation at 37° C for 30 minutes and centrifugation at 3000 rpm for (10–15) minutes. The supernatant's hemoglobin concentration was measured at 560 nm.[33] The equation for calculating protection (%) and hemolysis (%) was as follows:

$$\%\,Hemolysis = \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times 100$$

$$\%\,protection = \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times 100$$

Inhibition of Protein Denaturation

Human Albumin Inhibits Protein Denaturation in an Anti-Inflammatory Test

Using a method slightly different from another study, human albumin-prevented protein denaturation was employed to examine the anti-inflammatory properties of SIF-ME. The reaction solution (5 mL) contains phosphate-buffered saline at pH 6.4 (2.8 mL), human albumin (2.5 mL), and extract (0.5 mL) at varying concentrations of 1000, 500, 250, 125 and 62.5 µg/mL. Distilled water was utilized as a control. The solutions were incubated for 15 minutes at 37 ± 2 °C following a 5-minute heating at 70 °C. After cooling, the water in a vehicle was used as the sample, and the strength of absorption was recorded at 660 nm.[34]

The percentage inhibition (%) was calculated using a specific formula after the test was conducted three times:

$$\%\,Inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100$$

Xyline-Induced Ear Edema

Each batch of (n = 5) Swiss albino mice received treatment. Oral treatments for the animals included 4 groups: control group, normal saline (3 mL/kg), standard group, diclofenac sodium (10 mg/kg), and two treated groups: extract (100 and 200 mg/kg body weight).1 hour later, to induce edema 20 µL of xylene was administered to both sides of each mouse group's right ear. While the left ear served as the control. After fifteen minutes, the animals were put to sleep using chloroform anesthesia, and their ears were removed, measured, and weighed using a 3 mm Cork borer. The anti-inflammatory effect was quantified as a percentage reduction in edema in test animals versus the control group.[35]

$$(\%)\ inhibition\ of\ ear\ thickness = \frac{Average\ ear\ thickness\ (Control - test)}{Average\ ear\ thickness\ control} \times 100$$

Anti-Oxidant Activity

The free radical scavenging activity of the SIF-ME was analyzed using the 2.2-diphenyl-1-picryl-hydroxyl (DPPH) following.[36] The DPPH solution was prepared in 4 mg DPPH and 100 mL methanol, mixed with extract concentrations (250,500 and 1000 µg/mL) and 3.9 mL DPPH solution. Absorbance was measured at 517 nm with Ascorbic acid as the standard. The percentage of inhibition was calculated from triplicate measurement.

$$\%\,DPPH\ radical\ scavenging\ activity = \frac{\text{Absorbance of}(control - sample)}{Absorbance\ of\ control} \times 100$$

Computer-Aided Drug Designing (CADD)

Predict the Activity Spectra for Substances (PASS)

The structure of 18 compounds of SIF-ME was analyzed for analgesic, anti-inflammatory, and anti-oxidant studies using the PASS program. The tools predict a compound's activity spectrum as probable activity (Pa) or probable inactivity (Pi) (https://www.way2drug.com/passonline/) based on a structure-activity relationship (SAR). Pa and Pi values range from 0.000 to 1.000, where pa > pi explains that they are experimentally active. Pa > 0.7, suggesting rich medicinal activity.[37]

Molecular Docking

Protein Preparation

Crystal structure of target protein 5C1M (Mu-opioid), 6COX (cyclooxygenase-2), 2AZ5 (TNF-α), 2CKJ (Xanthine oxidoreductase) and 1R4U (Urate oxidase) with a selective inhibitor and with a resolution of 2.07 Å, 2.80 Å, 2.10 Å, 3.59 Å and 1.65 Å and was sourced from RCSB PDB (https://www.rcsb.org/) for studying plant biological activity. By using

Discovery Studio 2024 and Swiss-Pdb Viewer, as well as cleaning and other necessary preparations. The protein setup included gasteiger charge, energy minimization, and analysis with AMBER ffl4sB and gasteiger mode.[38]

Ligand Preparation

The 18 bioactive compounds of SIF-ME were 24-Noroleana-3,12-diene (PubChem CID: 15427754), S-Octahydro-9-phenanthrene methanol (PubChem CID: 607779), Epoxylathyrol (PubChem CID: 56841080), Retinoic acid (PubChem CID: 444795) were PubChem chemical database. The study compared the docking of SIF-ME phytocompounds with standards celecoxib, ibuprofen, and ascorbic acid to assess analgesic, anti-inflammatory, and anti-oxidant activity were studied to compare and juxtapose. Ligands were optimized using the PyRx for target suitability.[39]

Molecular Docking Analysis

In this study, PyRx Auto Dock Vina was employed, and protein and ligand structures were obtained and converted to PDBQT format. A grid box of dimensions: X: 67.7066 Å, Y: 71.9639 Å, and Z: 61.6788 Å was the center of the box to identify the best docking position, BIOVIA Discovery Studio Visualizer 2024 has been used for both two-dimensional (2D) and three-dimensional (3D) representations.[40]

In silico Study: Pharmacokinetic and Toxicological Study

The Swiss ADME was utilized to assess the pharmacokinetic parameters of the sort-out compounds (http://www.swissadme.ch/). Key drug-likeness parameters evaluated included molecular weight (MW), Hydrogen bond acceptors (HBA), Hydrogen bond donors (HBD), violations, Lipophilicity (LogP) following Lipinski's rules, and Total polar surface area (TPSA), Number of Rotatable bonds (nRB), based on Veber's rules.

Toxicological properties were analyzed utilizing the admetSAR tool (http://lmmd.ecust.edu.cn/), to address the concern regarding the toxicity discovery of the new drug. Ames toxicity, Carcinogenicity, Acute oral toxicity, Human Intestinal absorption, Bioavailability, and blood-brain barrier (BBB) were predicted.[41]

Eighteen compounds out of sixty bioactive compounds were selected for docking study, and forty-two compounds were rejected due to violation of Lipinski's, and Veber's rules and toxicological properties.[42,43]

Statistical Analysis

The study results were presented as mean ± SEM and using graph Pad prism 10, One-way analysis (ANOVA) was followed by Dunnett's test. Values were compared to the control group with statistical significance when *p < 0.05, **P<0.01 and ***P< 0.001. SEM = Standard error mean.

Results

Yields Percentage

After the extraction process, the percentage yields of SIF-ME was calculated to be 14.26%.

Qualitative Analysis

Qualitative screening of SIF-ME revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, tannins, phenols, gum, ash, and saponin, as shown in Table 1.

Quantitative Analysis

According to the results, the SIF-ME had promising levels of TPC, TFC, and TTC. The result has been expressed in Table 2.

Toxicity Evaluation

Based on this finding, the optimum dosage was established at (100 and 200 mg/kg body weight) for the in-vivo analgesic and inflammatory investigation. No harmful effects of the extract at the mentioned dosages were observed throughout this investigation.

Acute Oral Toxicity

General Sign and Behavioral Interpretation

The toxic impact of the SIF-ME on the visual appearance and the general behavioral pattern of 14 h (Table 3). No toxicological signs or deaths were detected in any of the animals that sustained up to 14 days after being administered the extract at a single dose of 500 mg/kg body weight. However, a single dose of 1000 mg/kg showed slight toxicity, and 2000 mg/kg showed toxicity and mortality Table 4.

GC-MS/MS Analysis

Upon GC-MS/MS analysis, approximately 60 compounds were identified in SIF-ME, with peak areas ranging from 0.15 to 8.29, detailed in Table 5, accompanied by chromatogram display Figure 1. The major compounds were 4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol (8.29%), 9-Octadecenamide (7.39%), 9,12,15-Octadecatrienoic acid, methyl ester (6.02%), 24-Noroleana-3,12-diene (5.93%), 9,12,15-Octadecatrienoic acid, 2.3-dihydroxypropyl ester, (5.34%), Sinapyl alcohol (3.68%), Beta -Sitosterol (3.5%), Methyl 5,11,14-eicosatrienoate (3.35%), Retinoic acid (2.57%). The rest of the compounds had a packed area of less than 2%.

Table 1 Qualitative Analysis of SIF-ME by the Interference of Phytochemical Constituents (Alkaloid, Carbohydrate, Flavonoid, Glycoside, Tannin, Steroid, Gum, Ash, and Saponin)

Phytochemical Constituents	Specific test	Inference
Alkaloids	Mayer's test	+
	Hager Test	+
	Wagner test	+
Carbohydrates	Molisch's test	+
	Benedict's test	+
	Fehling's test	+
Flavonoids	Alkaline reagent test	+
Gum	Alcohol test	+
Ash	H_2SO_4 + NaOH	+
Phenols	Ferric chloride test	+
Saponin	Foam test	+
Tannins	Gelatin test	+
Glycoside	Liebermann's test	+

Note: Symbol (+) indicates the presence of phytochemical constituents.

Table 2 Total Phenolic Content, Total Flavonoid Content, and Total Tannin Content of Examined SIF-ME

Total Phenolic Content (TPC) mg/g GAE	Total Flavonoid Content (TFC) mg/g	Total Tannin Content (TTC) mg/g
16.26 ± 0.67	9.27 ± 0.20	4.78 ± 0.34

Abbreviation: GAE, Gallic acid equivalent.

Table 3 General Appearance and Behavioral Observations for Control and Treated Groups for 14 Hours (100, 200, 400, 500, 1000 and 2000 Mg/Kg)

Observation	Control Group	100 mg/kg	200 mg/kg	400 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Slightly Red	Ruddy
Mucous membrane	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Behavioral patterns	Normal	Normal	Normal	Normal	Rapid Heart Beat	Rapid Heart Beat	Rapid Heart Beat
Salivation	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.	N.O
Lethargy	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.	Observed
Sleep	N.O.	N.O.	N.O.	N.O.	Observed	Observed	N.O.
Diarrheal	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
Coma	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
Tremors	N.O.	N.O.	N.O.	N.O.	N.O.	Observed	N.O.

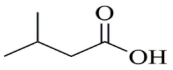
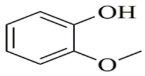
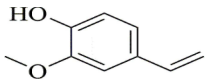
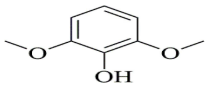
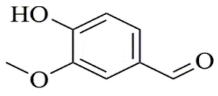
Abbreviation: N.O. indicates "Not Observed".

Table 4 Acute Oral Toxicity Effects (n=5) of SIF-ME in Mice Model for 14 Days

Control	Treatment group (500 mg/kg crude extract)	Treatment group (1000 mg/kg crude extract)	Treatment group (2000 mg/kg crude extract)
0 mortality	0 mortality	0 mortality	3 mortality cases recorded

Note: (n=5) indicates the number of mice used.

Table 5 Quantitative Compounds Identified from SIF-ME by GC-MS/MS Analysis

SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
1	Butanoic acid, 3-methyl	102.1 g/mol	3.904	0.15	
2	Phenol, 2-methoxy	124.1 g/mol	6.83	0.37	
3	2-Methoxy-4-vinyl phenol	150.1 g/mol	9.289	0.29	
4	Phenol, 2.6-dimethoxy	154.1 g/mol	9.632	0.38	
5	Vanillin	152.1 g/mol	10.171	0.3	

(*Continued*)

Table 5 (Continued).

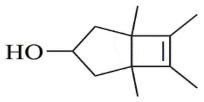
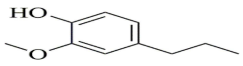
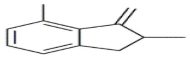
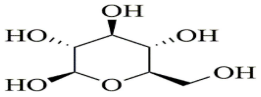
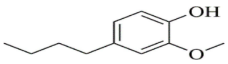
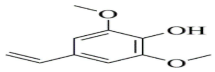
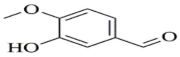
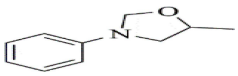
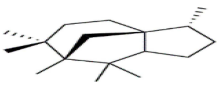
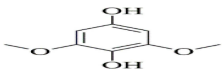
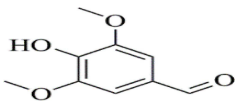
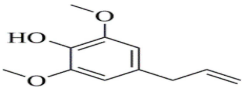
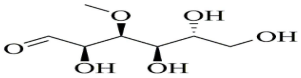
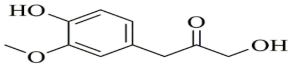
SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
6	endo-1,5,6,7-Tetramethylbicyclo[3.2.0]hept-6-en-3-ol	166.2 g/mol	10.626	0.8	
7	Phenol, 2-methoxy-4-propyl	166.2 g/mol	10.695	0.24	
8	3(2H)-Benzofuranone, 2.4-dimethyl	162.1 g/mol	10.971	0.2	
9	beta.-D-Glucopyranose, 1.6-anhydro	162.1 g/mol	11.031	0.22	
10	Guaiacol, 4-butyl	180.2 g/mol	11.35	0.55	
11	Phenol, 4-ethenyl-2,6-dimethoxy	180.2 g/mol	11.663	0.33	
12	Benzaldehyde, 3-hydroxy-4-methoxy	152.1 g/mol	11.934	0.28	
13	5-Methyl-3-phenyl-1,3-oxazolidine	163.2 g/mol	12.375	0.41	
14	(3R,3aS,6S,7R)-3,6,8,8-Tetramethyloctahydro-1H-3a,7-methanoazulen-6-ol	222.3 g/mol	12.425	0.17	
15	2,6-Dimethoxyhydroquinone	170.1 g/mol	12.602	0.32	
16	Benzaldehyde, 4-hydroxy-3,5-dimethoxy	182.1 g/mol	12.708	0.49	
17	Phenol, 2.6-dimethoxy-4-(2-propenyl)	194.2 g/mol	13.18	0.49	
18	3-O-Methyl-d-glucose	194.1 g/mol	13.251	0.29	
19	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)	196.2 g/mol	13.566	0.93	

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Table 5 (Continued).

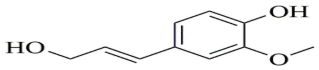
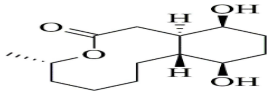
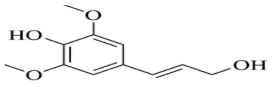
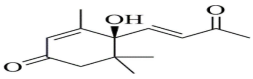
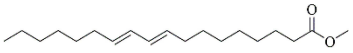
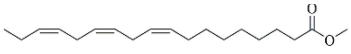
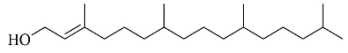
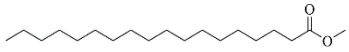
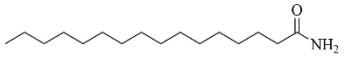
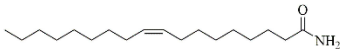
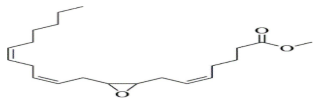
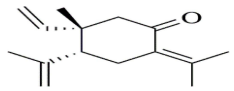
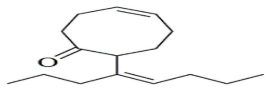
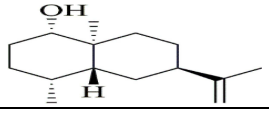
SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
20	4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenyl	180.2 g/mol	13.747	8.29	
21	Dihydroxy-4-methyldodecahydro-2H-benzo[d] oxecin-2-one	256.3 g/mol	14.882	0.38	
22	Sinapyl alcohol	210.2 g/mol	17.386	3.68	
23	4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl) cyclohex-2-enone	222.2 g/mol	18.661	0.25	
24	9,11-Octadecadienoic acid, methyl ester	294.5g/mol	18.941	2.56	
25	9,12,15-Octadecatrienoic acid, methyl ester	292.5 g/mol	19.046	6.02	
26	Phytol	296.5 g/mol	19.197	0.97	
27	Methyl stearate	298.5 g/mol	19.449	0.67	
28	Hexadecanamide	255.4 g/mol	20.421	0.39	
29	9-Octadecenamide	281.5g/mol	23.448	7.39	
30	Z,Z,Z-8,9-Epoxyeicosa-5,11,14-trienoic acid, methyl ester	334.5 g/mol	23.558	1.54	
31	Cyclohexanone, 5-ethenyl-5-methyl-4-(1-methylethenyl)-2-(1-methylethylidene)	218.3 g/mol	23.75	0.75	
32	4-Cycloocten-1-one, 8-(4-octen-4-yl)	234.3 g/mol	23.876	0.93	
33	5.beta.,7.beta.H,10.alpha.-Eudesm-11-en-1.alpha.-ol	222.3 g/mol	23.99	0.71	

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Table 5 (Continued).

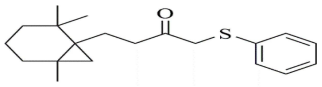
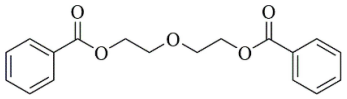
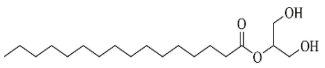
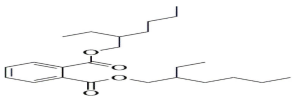
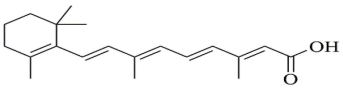
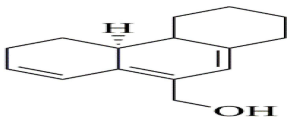
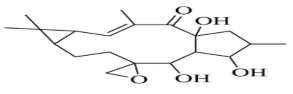
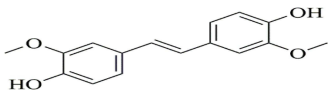
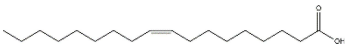
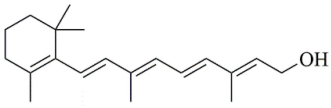
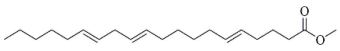
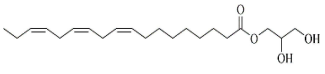
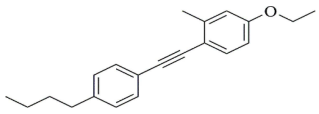
SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
34	Bicyclo[4.1.0]heptane, 1-(3-oxo-4-phenylthiobutyl)-2,2,6-trimethyl	316.5 g/mol	25.15	0.58	
35	Diethylene glycol dibenzoate	314.3g/mol	25.397	0.39	
36	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	330.5g/mol	25.849	0.64	
37	Bis(2-ethylhexyl) phthalate	390.6g/mol	26.147	0.31	
38	Retinoic acid	300.4 g/mol	26.94	2.57	
39	S-Octahydro-9-phenanthrene methanol	216.3 g/mol	27.12	1.03	
40	Epoxylathyrol	350.4 g/mol	28.065	0.61	
41	3,3'-Dimethoxy-4,4'-dihydroxystilbene	272.2 g/mol	28.195	0.6	
42	Oleic Acid	354.6 g/mol	28.38	0.39	
43	Retinol	286.5 g/mol	28.44	1.34	
44	Methyl 5,11,14-eicosatrienoate	320.5 g/mol	28.636	3.35	
45	9,12,15-Octadecatrienoic acid, 2.3-dihydroxypropyl ester, (Z,Z,Z)-	352.5 g/mol	28.755	5.34	
46	Benzene, 1-[(4-butyl phenyl)ethynyl]-4-ethoxy-2-methyl-	292.4 g/mol	28.83	2.59	

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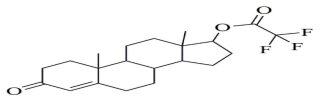
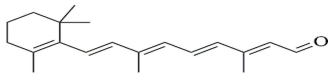
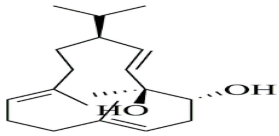
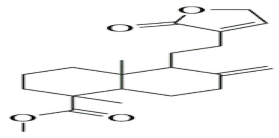
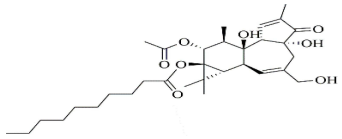
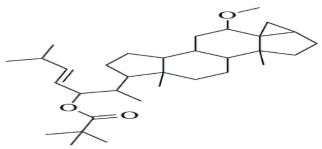
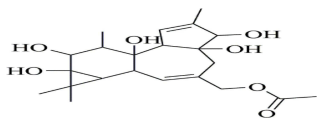
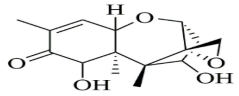
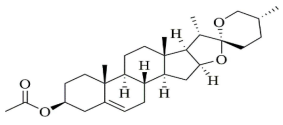
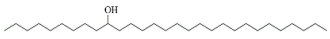
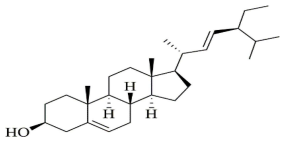
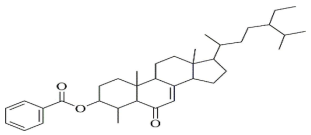
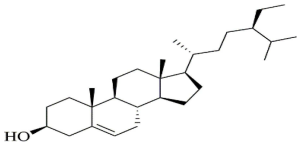
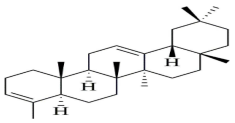
Table 5 (Continued).

SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
47	10,13-Dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl 2,2,2-trifluoroacetate	331.5 g/mol	28.974	3.04	
48	Retinal	284.4 g/mol	29.323	1.04	
49	(1S,2E,4S,5R,7E,11E)-Cembra-2,7,11-trien-4,5-diol	306.5 g/mol	29.41	0.58	
50	Methyl 1,4a-dimethyl-6-methylidene-5-[2-(5-oxo-2H-furan-4-yl)ethyl]-3,4,5,7,8,8a-hexahydro-2H-naphthalene-1-carboxylate	346.5 g/mol	29.72	1.73	
51	9-(Acetyloxy)-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9aH-cyclopropa	560.7 g/mol	30.861	1.7	
52	Cholest-22-ene-21-ol, 3.5-dehydro-6-methoxy-, pivalate	498.8 g/mol	31.266	1.96	
53	1H-Cyclopropa[3,4]benz[1,2-e]azulene-4a,5,7b,9,9a(1aH)-pentol, 3-[(acetyloxy)methyl]-1b,4,5,7a,8,9-hexahydro-1,1,6,8-tetramethyl	492.6 g/mol	31.842	2.44	
54	(11.xi.)-4,7-Dihydroxy-12,13-epoxytrichothec-9-en-8-one	280.3 g/mol	33.31	0.22	
55	Spirost-5-en-3-ol, acetate, (3.beta.,25R)-	456.7 g/mol	34.002	0.24	

(*Continued*)

Table 5 (Continued).

SL	Compounds	MW(g/mol)	Retention Time	Area %	Structure
56	Nonacosan-10-ol	424.8 g/mol	34.551	0.73	
57	Stigmasterol	412.7 g/mol	37.316	1.18	
58	22-Desoxycarpesterol	546.8 g/mol	37.815	0.33	
59	beta-Sitosterol	414.7 g/mol	38.573	3.5	
60	24-Noroleana-3,12-diene	394.7 g/mol	39.627	5.93	

Analgesic Activity

Hot Plate Test

The results are expressed in Table 6; the results are significantly extended dose-dependent reaction times of heat sensation ($p < 0.001$) compared to the negative control at the doses of 100 and 200 mg/kg BW. The 60-minute observation result showed

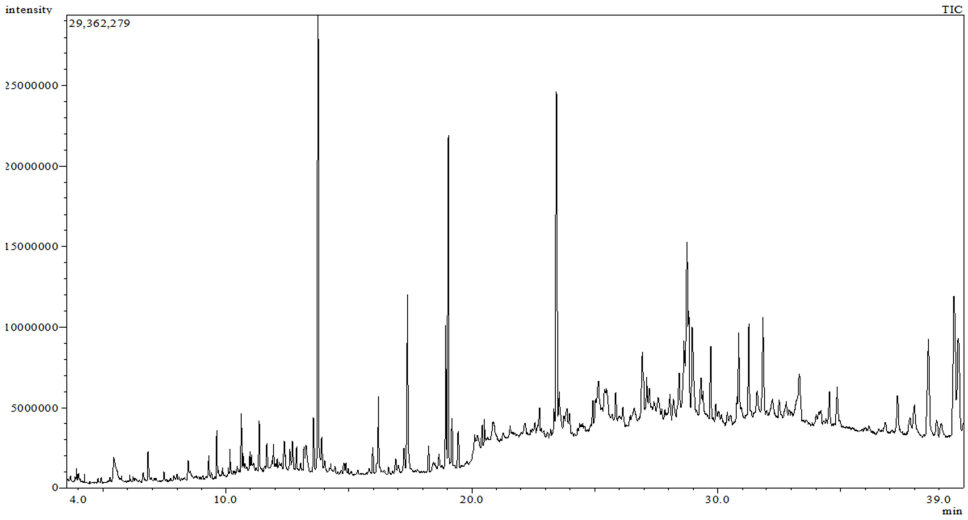


Figure 1 GC-MS chromatograph of methanolic extract of *Shirakiopsis indica* fruits.

Table 6 Analgesic Activity of SIF-ME by Hot Plate Method in Different Time Peaks (0, 30, 60 and 120 Min)

Group	Dose	Response time (sec)				% Inhibition		
		0 min	30 min	60 min	120 min	30 min	60 min	120 min
Control	10 mL/kg	7.80 ± 0.37	9.3 ± 0.43	6.76 ± 0.26	8.64 ± 0.36	–	–	–
Diclofenac-Na	10 mg/kg	12.48 ± 0.65	16.95 ± 0.37***	15.65 ± 0.30***	17.89 ± 1.02***	36.96	38.25	43.3
SIF- ME	100 mg/kg	10.62 ± 0.96	12.94 ± 0.26**	14.73 ± 0.62**	12.06 ± 0.41**	17.58	34.29	16.01
SIF-ME	200 mg/kg	8.69 ± 0.54	15.76 ± 0.33***	16.73 ± 0.35***	19.75 ±1.45***	31.21	32.5	52.01

Notes: One way Analysis of variance (ANOVA) followed by Dunnett's test was performed as the significance test. All values are Mean ± SEM (n=5). ***$p<0.001$, **$p< 0.01$, compared to control group.

that the reaction time at doses 100 and 200 mg/kg expanded to 34.29% and 32.5%, respectively, whereas the standard drug showed 38.24%. However, in the 120-minute observation, 200 mg/kg doses were prolonged, and the reaction time was 52.01%, whereas the standard drug and 100 mg/kg doses declined (43.3% and 16.1%) Figure 2.

Acetic Acid-Induced Writhing Test

Table 7 displays the inhibition of the leaking response in mice administered the test medications during the acetic acid-induced writhing test. Mice's abdominal writhes were dramatically ($p < 0.001$) reduced when both dosages of SIF-ME were given orally. This effect was dose-dependent, delineated in Figure 3. The extract exhibited a percentage inhibition of the writhing response of 17.39% and 43.47% at doses 100 and 200 mg/kg. In contrast, the standard dose of diclofenac sodium (10 mg/kg) demonstrated 81.52% inhibition relative to the control.

Formalin-Induced Licking Test

The extract of SIF-ME demonstrated significant inhibition of licking response in mice during the formalin-induced pain test, as shown in Table 8. It notably reduces licking by 38.82% at 100 mg/kg, 43.3% at 200 mg/kg in the early phase, and by 53.94% at 100 mg/kg and 61.84% at 200 mg/kg in the late phase, displayed in Figure 4. Both of these inhibitions were dose-dependent and comparable to standard drugs:

Anti-Inflammatory Activity

HRBC Membrane Stabilizing Assay

The HRBC membrane stabilizing assay evaluated the anti-inflammatory effect of SIF-ME, revealing significant findings in Table 9. The extract demonstrated substantial protection at 1000 µg/mL concentration with 84.08% and minimal

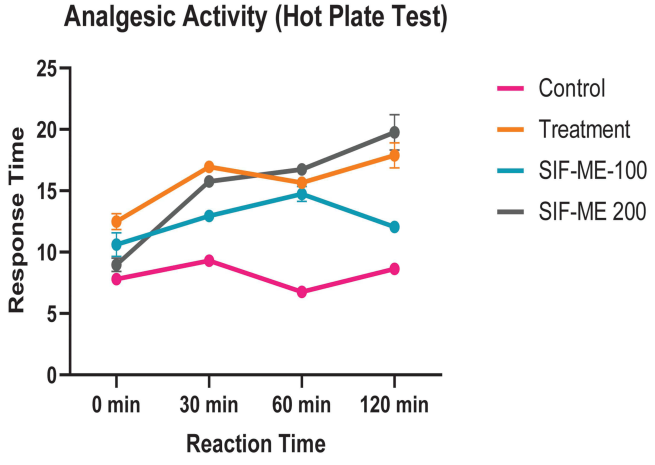


Figure 2 Analgesic activity observation of SIF-ME by hot plate comparison method in different time points (0, 30, 60, and 120 min).

Table 7 Acetic Acid-Induced Writhing Test Results Were Examined on Mice in Different Test Groups (Control, Standard, 100 and 200 Mg/Kg)

Groups	Treatment	Dose and Route	No. of Writhing	% of Writhing	% Inhibition
G-I	1% Tween 80(control)	10 mL/kg; p.o	30.66 ± 0.88	100	NA
G- II	Diclofenac Na (Standard)	10 mg/kg; p.o	5.66 ± 0.6***	18.46	81.52
G- III	SIF-ME	100 mg/kg; p.o	20.33 ± 0.88**	82.61	17.39
G- IV	SIF-ME	200 mg/kg; p.o	17.33± 0.66***	46.73	43.47

Notes: One way Analysis of variance (ANOVA) followed by Dunnett's test was performed as the significance test. All values are Mean ± SEM (n=5). ***p<0.001, **p< 0.01, compared to control group.

hemolysis of 16.91%. Conversely, 62.5 µg/mL concentration, lower protection (49.41%), and higher hemolysis (50.58%). These results are compared to standard drugs, which are superior at 1000 µg/mL with 95.60% protection and only 4.39% hemolysis (Figure 5A and B).

Inhibition of Protein Denaturation

The in-vitro anti-inflammatory test for SIF-ME showed a mean inhibition percentage of protein denaturation of 67.34, 63.12, 37.04, 29.45, and 26.05% for concentrations of 1000, 500, 250, 125, and 62.5 µg/mL. In the case of diclofenac-Na, the percentage of inhibition was 88.77, 83.96, 78.75, 69.19, and 62.12% at the same concentration Table 9. The results showed that the standard group exhibited a maximum inhibition percentage of 88.77% at a concentration of 1000 µg/mL. In comparison, the SIF-ME extract demonstrated a prominent inhibition percentage of 67.34% at the same concentration. From the graph, it was found that the ability of the SIF-ME extract was statistically significant (Figure 5C).

Xylene-Induced Ear Edema

In the study, on xylene-induced ear edema (Table 10), the dosages of 100 and 200 mg/kg exhibited significant inhibitory effects. Resulting in percentage inhibitions of 40.19% and 54.07%, respectively. The highest inhibitory effect at a dosage of 200 mg/kg was comparable to that of Diclofenac sodium, with an inhibition of 74.28% (Figure 5D).

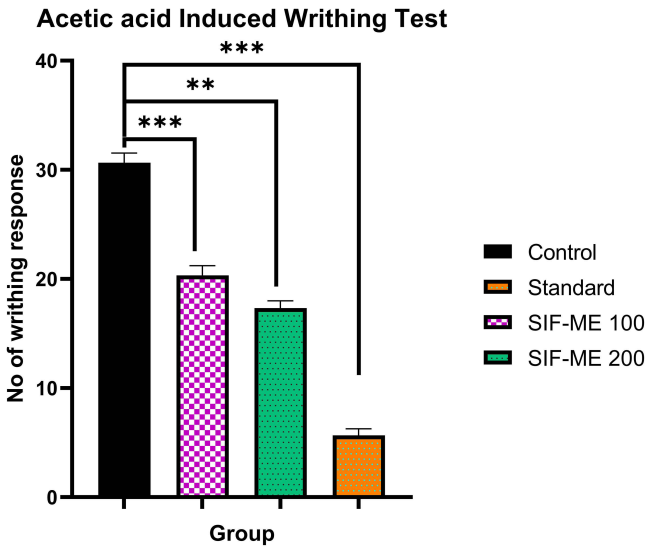


Figure 3 Numbers of writhing responses in analgesic acetic acid-induced writhing test in control, standard, and test sample groups (Control, standard, 100 and 200 mg/kg) (***p<0.001, **p< 0.01, compared to the control group).

Table 8 Percentage Inhibition of SIF-ME by Formalin-Induced Licking Test of Different Concentrations in Test Groups (Control, Standard, 100 and 200 Mg/Kg) at Early and Late Phases

Groups	Treatment	Dose and Route	Early phase (0–5min)		Late Phase(20–30min)	
			Licking time (s)	Inhibition %	Licking time(s)	Inhibition %
G-I	1% Tween 80 (control)	10 mL/kg; p.o	22.33 ± 0.66	NA	25.33 ± 1.73	NA
G- II	Diclofenac Na (Standard)	10 mL/kg; p.o	10.33 ± 0.88***	53.73	6.55 ± 0.33***	74.34
G- III	SIF-ME	100 mg/kg; p.o	13.66 ± 0.88**	38.82	11.66 ± 0.66**	53.94
G- IV	SIF-ME	200 mg/kg; p.o	12.66 ± 0.33**	43.3	9.66 ± 1.20**	61.84

Notes: All values are Mean ± SEM (n=5). One way Analysis of variance (ANOVA) followed by Dunnett's test was performed as the significance test. All values are Mean ± SEM (n=5). ***p<0.001, **p< 0.01, compared to control group.

Anti-Oxidant Activity

DPPH Scavenging Assay

The results are visualized in (Figure 6) as a concentration curve, with a SIF-ME 1000 µg/mL concentration exhibiting the highest inhibition percentage of 74.70% ± 3.92%. In comparison, ascorbic acid demonstrated higher inhibition (96.28% ± 0.44%) at 1000 µg/mL. The IC_{50} value for SIF-ME is 469.5 µg/mL. This suggests that this extract concentration has a moderate impact against DPPH compared to the standard drug ascorbic acid 10.12 µg/mL.

Computer Aided Drug Design

In-Silico Pass Prediction

Eighteen isolated compounds underwent analysis using the PASS online tool to assess the analgesic, anti-inflammatory, and antioxidant activities of the plant. The potent showed higher Pa values than Pi values for both analgesic, anti-inflammatory and anti-oxidant activity. Whereas Eight compounds (Butanoic acid, 3-methyl-; Phenol, 2.6-dimethoxy-; Vanillin; Phenol, 2-methoxy-4-propyl-; 2.6-Dimethoxyhydroquinone, 2-Propanone; 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-; Octahydro-

Figure 4 Total licking response of formalin-induced licking test (early phase and late phase) in control, standard, and test groups (100 and 200 mg/kg) (***p<0.001, **p< 0.01, compared to the control group).

Table 9 HRBC Membrane Stabilizing and Protein Denaturation Result of SIF-ME Compared to Standard in Different Concentrations (62.5, 125, 250, 500 and 1000 µg/Ml)

Sample	Concentration (µg/mL)	HRBC membrane stabilization			Protein Denaturation	
		Mean ± SEM	% Protection	% Hemolysis	Mean ± SEM	% Inhibition
SIF-ME	62.5	3.05 ± 0.05^{ns}	49.41	50.58	3.69 ± 0.2^{ns}	26.05
	125	2.05 ± 0.05^{ns}	66.03	33.99	3.52 ± 0.19^{ns}	29.45
	250	1.92 ± 0.54^{ns}	68.03	31.84	3.14 ± 0.4^{ns}	37.04
	500	1.35 ± 0.14^{ns}	77.61	22.38	1.84 ± 0.04^{ns}	63.12
	1000	1.02 ± 0.19^{ns}	83.08	16.91	1.60 ± 0.19^{ns}	67.34
Standard (Diclofenac-Na)	62.5	1.93 ± 0.2^{ns}	68.49	31.50	1.89 ± 0.07^{ns}	62.12
	125	1.56 ± 0.20^{ns}	74.12	25.87	1.54 ± 0.13^{ns}	69.19
	250	1.03 ± 0.21^{ns}	82.91	17.08	1.06 ± 0.11^{ns}	78.75
	500	0.53 ± 0.06^{ns}	91.11	9.48	0.80 ± 0.06^{ns}	83.96
	1000	0.26 ± 0.08^{ns}	95.60	4.39	0.56 ± 0.07^{ns}	88.77

Notes: One way Analysis of variance (ANOVA) followed by Dunnett's test was performed as the significance test. All values are Mean ± SEM (n=3). ^{ns}P = non-significant as compared with a standard group.

9-phenanthrene methanol; 24-Noroleana-3,12-diene) showed both analgesic, anti-inflammatory and anti-oxidant activity. Among the compounds, 24-Noroleana-3,12-diene showed the highest Pa value in both analgesic, anti-inflammatory, and anti-oxidants (0.726, 0.848, and 0.280). The outcome is presented in Table 11.

Molecular Docking Analysis

The molecular docking investigation of SIF-ME is outlined in Table 12. The docking assessment of the active compounds was subjected to the active site 5C1M (Mu-opioid) and 6COX (cyclooxygenase-2) for analgesic enzymes, 2AZ5 (TNF-α) for anti-inflammatory enzymes and 2CKJ (xanthine oxidoreductase), 1R4U (urate oxidase) for anti-oxidant receptor. Grid docking analysis was performed using PyRx AutoDock Vina to study the interaction of all 18 compounds with standards. Our study has shown that 24-Noroleana-3,12-diene have the highest binding affinity against analgesic, anti-inflammatory and anti-oxidant activity (−10.5 against 5C1M, −10.4 against 6COX, −9.4 against 2AZ5, −9.4 against 2CKJ, −8.9 against 1R4U respectively) Compared with standard drug celecoxib, ibuprofen and ascorbic acid (−10.3 against 5C1M, −10 against 6COX, −6.2 against 2AZ5, −6.5 against 2CKJ, −5.4 against 1R4U). Graphical represented (2D and 3D) whose compounds are represented highest binding affinity with standard (Figure 7–11).

ADME/T-Analysis

All the phytocompounds were taken for ADME/T evaluation and drug likeliness. Out of 60 compounds, 18 compounds do not violate Lipinski's rule of five and Veber's rules, and 42 compounds violate Lipinski's rules and Veber's rules.

All the phytocompounds were subjected to further toxicity tests, where 18 compounds passed the criteria. The compounds were screened on Ames toxicity, acute oral toxicity, carcinogenesis, and blood-brain barrier permeability, where the compounds showed a high GI absorption rate and optimum solubility. Among the 18 compounds, only S-Octahydro-9-phenanthrene methanol had Ames toxicity. All the other compounds followed Lipinski's rule of five. For the data screening, AdmetSAR was utilized for toxicity evaluation, with celecoxib, ibuprofen, and ascorbic acid as the standard (Table 13).

Discussion

Salicylate-containing plants were used for medicinal purposes over a prolonged duration, resulting in the development of a significant anti-inflammatory medication known as aspirin. Aspirin, a compound possessing anti-inflammatory

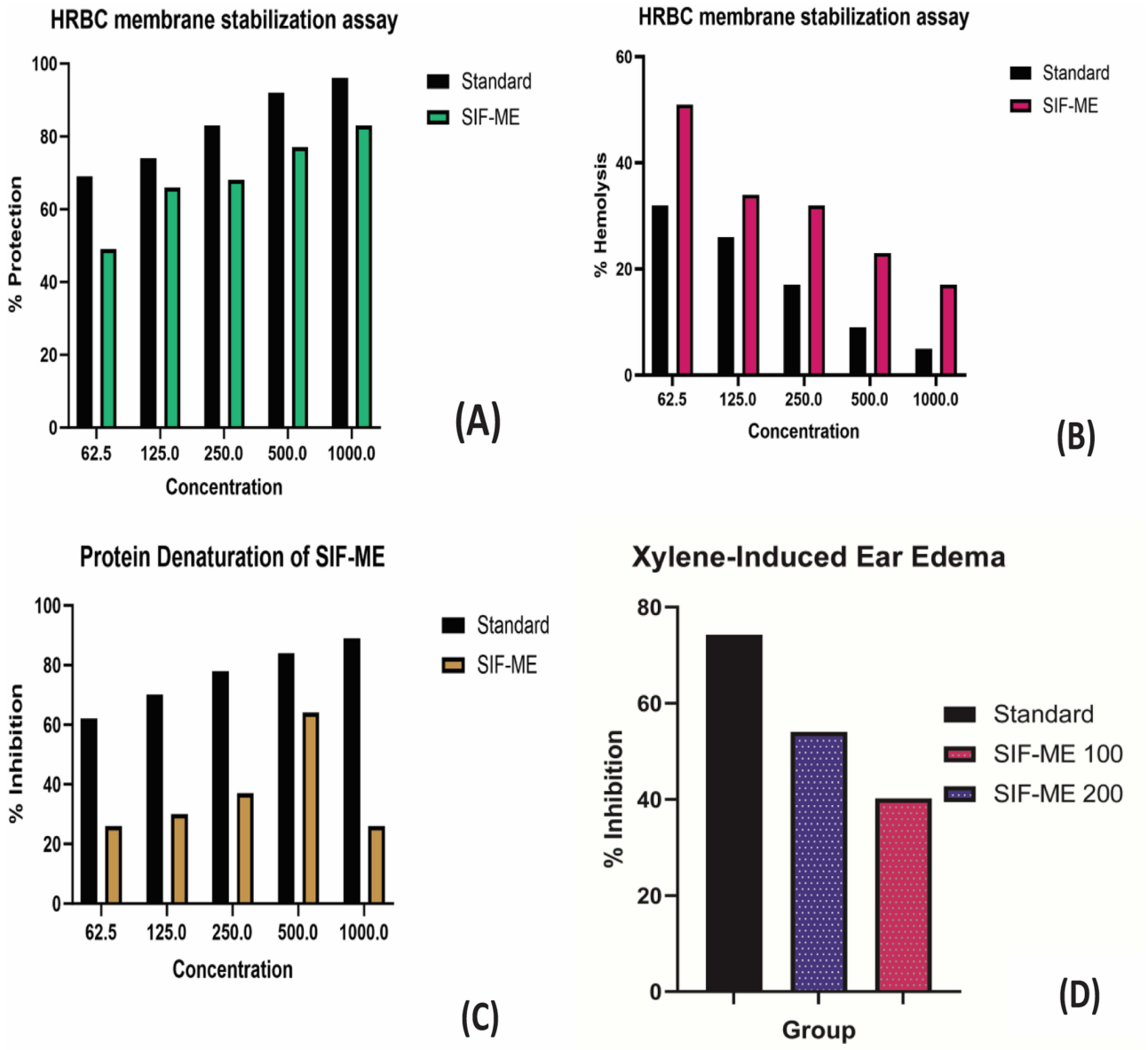


Figure 5 (**A**) Percentage protection of HRBC membrane stabilization assay in different concentrations (62.5, 125, 250, 500 and 1000 µg/mL). (**B**) Percentage hemolysis of HRBC membrane stabilization assay in different concentrations (62.5, 125, 250, 500 and 1000 µg/mL). (**C**) Percentage inhibition of protein denaturation of SIF-ME in different concentrations (62.5, 125, 250, 500 and 1000 µg/mL). (**D**) Xylene-induced ear edema percentage inhibition in different concentrations (Standard, 100 and 200 mg/kg).

properties, is obtained from natural sources and is widely utilized in modern healthcare.[44] Within the realm of Bangladeshi traditional medicine, a diverse range of natural ingredients and formulations have been employed to mitigate pain, inflammation and oxidative stress.[45] Reactive oxygen species (ROS) encompass free radicals. Severe quantities of ROS can lead to considerable harm to many molecules such as proteins, lipids, RNA, and DNA, due to their strong reactivity[46] This work is the initial comprehensive assessment of the effectiveness of SIF-ME, combining pharmacological and phytochemical compounds in treating peripheral (writhing) antinociceptive, anti-inflammatory, and antioxidant activities. It is followed by a computational study of its bioactive compounds.

In this study, the pain-relieving effect of SIF-ME was evaluated utilizing thermal and chemical methods to induce nociceptive pain in mice. The acetic acid-induced writhing test, a well-established model for peripheral nociception in mice.[47] Peripheral pain relief may involve inhibiting cyclooxygenases and/or lipoxygenases, whereas central pain relief is a central pain receptor inhibitor. The hot plate test, heat-induced assessing centrally mediated pain, and acetic-acid test

Table 10 Xylene-Induced Ear Edema Test Result of Several Concentrations (Control, Standard, 100 and 200 Mg/Kg)

Treatment	Dose	Weight of Right Ear (mg)	Weight of left Ear (mg)	Difference (mg)	Inhibition %
Control	3 mL/kg	13.4 ± 1.1	3.1 ± 0.5	10.3 ± 1.6	NA
Standard (Diclofenac-Na)	10 mL/kg	5.05 ± 0.25	2.55 ± 0.35	2.5 ± 0.6***	74.28
SIF-ME	100 mg/kg	9.2 ± 0.68	3.03 ± 0.31	6.16 ± 0.3*	40.19
SIF-ME	200 mg/kg	7.43 ± 4.29	2.7 ± 0.2	4.73 ± 0.4**	54.07

Notes: All values are Mean ± SEM (n=5). One way Analysis of variance (ANOVA) followed by Dunnett's test was performed as the significance test. All values are Mean ± SEM (n=5). ***p<0.001, **p< 0.01, *p< 0.05 compared to control group.

evaluate centrally and peripherally acting.[48,49] Diclofenac sodium is a non-opioid analgesic medication that inhibits the peripheral and central nervous systems; It's similar to that of aspirin. To examine the mechanism of the analgesic activity of SIF-ME, with diclofenac sodium as a reference drug.

The findings demonstrated that SIF-ME significantly suppressed the occurrence of writhes when compared to the control group (Figure 3). The previous study by Oyebenji et al showed that Strong anti-inflammatory and analgesic (pain-relieving) effects were demonstrated by the methanolic extract of *Stellaria media* leaf, which was mediated by both peripheral and central pathways.[50] Nevertheless, all test samples had no substantial impact in the hot-plate scenario. Collectively, the SIF-ME may have the capacity to inhibit the experience of pain in the acetic acid test through peripheral pain pathways rather than a central pathway.

The formalin-induced test served as a robust model for inflammatory and nociceptive pain.[51] The test comprises two phases: the first phase includes direct stimulation and the second phase exhibits peripheral inflammation and modification of central processing. It triggers a neurogenic response involving substance P and bradykinin in the first phase, with prostaglandins, histamine, NO, and serotonin released in the second phase.[52] Central-acting drug affects equally two phases while peripheral drugs affect the second phase.[53]

The experimental findings indicate that the SIF-ME generates an anti-nociceptive effect in both stages of the formalin-induced process. The extract showed an analgesic effect in both modes in both phases, but the second phase may act as an anti-inflammatory (Figure 4).[54]

SIF-ME demonstrated anti-inflammatory properties by preventing the breakdown of the red blood cell (RBC) membrane caused by hypotonicity. The results indicate that the extract has the potential to stabilize the lysosomal

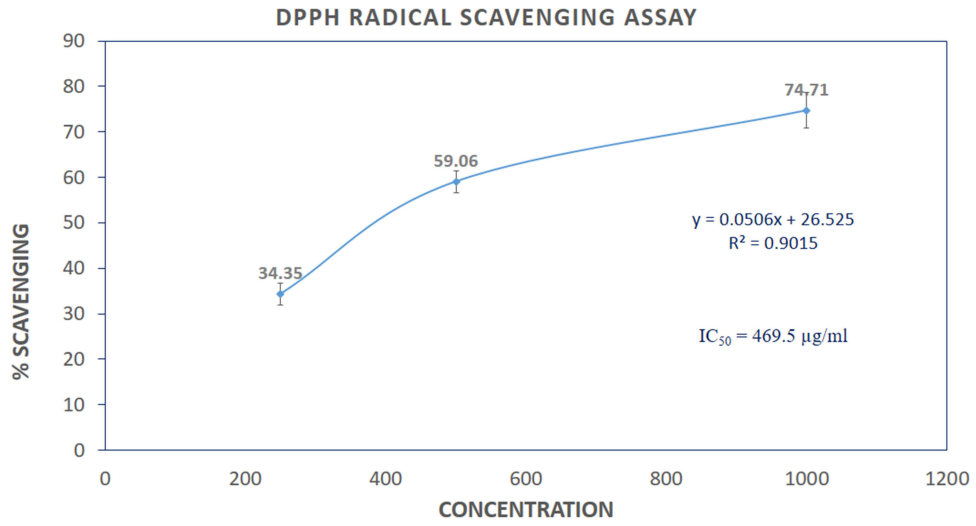


Figure 6 DPPH radical scavenging of SIF-ME. The results are expressed as a percentage of reducing activity equivalent to ascorbic acid. Values expressed as mean ± SEM with n=3.

Table 11 In silico Pass Prediction of Selected Bioactive Compounds Found on SIF-ME to Evaluate Analgesic, Anti-Inflammatory, and Anti-Oxidant Probability

Serial No	Compound	PASS Prediction for Analgesic activity		PASS Prediction for Anti-inflammatory Activity		PASS Prediction for Antioxidant activity	
		Pa	Pi	Pa	Pi	Pa	Pi
01	Butanoic acid, 3-methyl	0.369	0.009	0.406	0.009	0.558	0.005
02	Phenol, 2-methoxy	–	–	0.475	0.064	0.419	0.011
03	2-Methoxy-4-vinyl phenol	–	–	0.520	0.521	0.459	0.008
04	Phenol, 2.6-dimethoxy	0.267	0.074	0.446	0.074	0.435	0.009
05	Vanillin	0.267	0.074	0.446	0.074	0.403	0.012
06	Phenol, 2-methoxy-4-propyl	0.230	0.136	0.391	0.101	0.310	0.021
07	Phenol, 4-ethenyl-2,6-dimethoxy	–	–	0.514	0.053	0.456	0.008
08	Benzaldehyde, 3-hydroxy-4-methoxy	–	–	–	–	0.403	0.012
09	5-Methyl-3-phenyl-1,3-oxazolidine	–	–	–	–	–	–
10	2,6-Dimethoxyhydroquinone	0.250	0.099	0.489	0.060	0.566	0.005
11	Benzaldehyde, 4-hydroxy-3,5-dimethoxy	–	–	–	–	0.236	0.066
12	Phenol, 2.6-dimethoxy-4-(2-propenyl)	0.204	0.192	0.496	0.058	–	–
13	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)	0.232	0.131	0.718	0.014	0.272	0.029
14	Retinoic acid	–	–	–	–	0.652	0.004
15	Octahydro-9-phenanthrene methanol	0.311	0.031	0.371	0.111	0.219	0.046
16	Epoxylathyrol	–	–	–	–	–	–
17	Beta –Sitosterol	–	–	0.467	0.067	0.178	0.012
18	24-Noroleana-3,12-diene	0.726	0.003	0.848	0.005	0.280	0.027

Notes: "Pa" indicates "Probability to be active" while "Pi" indicates "Probability to be inactive".

Table 12 Molecular Docking Result of Selected Bioactive Compounds Found in SIF-ME and Standards Against Receptor

SL No	Compounds	CID	Binding Affinities (kcal/mol)				
			Analgesic (central)	Analgesic (peripheral)	Antioxidant		Anti-inflammatory
			Mu-opioid (5C1M)	COX-2 (6COX)	Xanthine oxidoreductase (2CKJ)	Urate oxidase (1R4U)	TNF-α (2AZ5)
01	Butanoic acid, 3-methyl	10430	−4	−4.6	−4.3	−3.9	−4.2
02	Phenol, 2-methoxy	460	−5	−5.6	−5.5	−5.3	−4.9
03	2-Methoxy-4-vinylphenol	332	−5.3	−6.5	−5.4	−5	−5.3
04	Phenol, 2.6-dimethoxy	7041	−5.2	−5.4	−5.5	−4.8	−4.7

(*Continued*)

Table 12 (Continued).

SL No	Compounds	CID	Binding Affinities (kcal/mol)				
			Analgesic (central)	Analgesic (peripheral)	Antioxidant		Anti-inflammatory
			Mu-opioid (5C1M)	COX-2 (6COX)	Xanthine oxidoreductase (2CKJ)	Urate oxidase (1R4U)	TNF-α (2AZ5)
05	Vanillin	1183	−5.3	−5.9	−5.6	−5.2	−5.2
06	Phenol, 2-methoxy-4-propyl	17739	−5.6	−5.7	−5.7	−5.1	−5.7
07	Phenol, 4-ethenyl-2,6-dimethoxy	35960	−5.8	−5.9	−5.5	−5.4	−5.3
08	Benzaldehyde, 3-hydroxy-4-methoxy	12127	−5.1	−5.9	−5.5	−5.3	−4.8
09	5-Methyl-3-phenyl-1,3-oxazolidine	319003	−5.8	−6.4	−5.6	−5.7	−5.6
10	2,6-Dimethoxyhydroquinone	96038	−51	−5.8	−5.7	−4.9	−4.9
11	Benzaldehyde, 4-hydroxy-3,5-dimethoxy	8655	−5.3	−5.6	−5.5	−5.4	−5
12	Phenol, 2.6-dimethoxy-4-(2-propenyl	226486	−5.9	−5.8	−5.8	−5.5	−5.8
13	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl	586459	−5.7	−6.2	−6.2	−5.7	−5.7
14	Retinoic acid	444795	−8.3	−8.3	−7.7	−7.1	−7.4
15	Octahydro-9-phenanthrene methanol	607779	−7.9	−7.9	−8	−7.2	−7.6
16	Epoxylathyrol	56841080	−8.2	−8.4	−8.2	−7.7	−8.6
17	Beta -Sitosterol	222284	−9.3	−9	−3.6	−7.7	−8.6
18	24-Noroleana-3,12-diene	15427754	−10.5	−10.4	−9.4	−8.9	−9.4
Standards	Celecoxib	2662	−10.3	−10	–	–	–
	Ibuprofen	3672	–	–	–	–	−6.2
	Ascorbic acid	54670067	–	–	−6.5	−5.4	–

5C1M (Mu-opioid) and 6COX (cyclooxygenase-2) and standard celecoxib for analgesic enzymes, 2AZ5 (TNF-α) and standard ibuprofen for anti-inflammatory enzymes while 2CKJ (xanthine oxidoreductase), 1R4U (urate oxidase) and standard ascorbic acid for anti-oxidant enzymes.

membrane. Inflammatory triggers the potential harm to tissue. Thus, maintaining lysosomal stability is essential for controlling the inflammatory reaction. SIF-ME can potentially hinder these activities and enhance the removal of intracellular components. The differences in anti-inflammatory activity between SIF-ME and the standard (Diclofenac-Na) were statistically non-significant, indicating that SIF-ME was beneficial for anti-inflammatory activity.

The protein denaturation technique of human albumin was selected to assess the anti-inflammatory characteristics of SIF-ME. The protein-denaturation test involves the detection of human albumin through thermal treatment.[55] The denatured protein produced antigens linked to certain diseases.[56] Proteins that have been denatured by heat are equally potent as proteins in their natural state in causing delayed hypersensitivity reactions. Typically, NSAIDs reduce

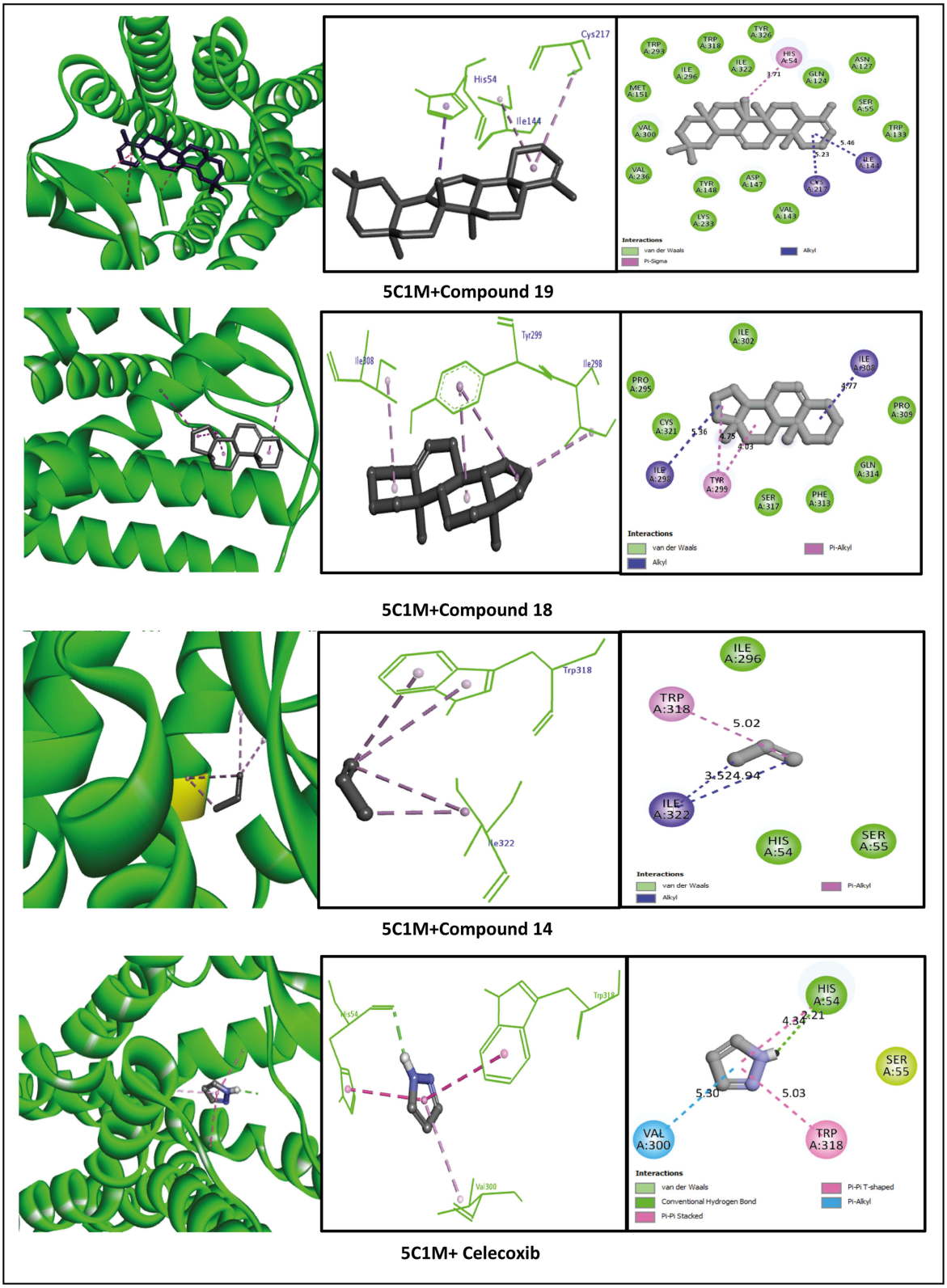


Figure 7 Graphical representation of the molecular interactions of the most prominent bioactive with the 5C1M enzyme with 3D visualization.

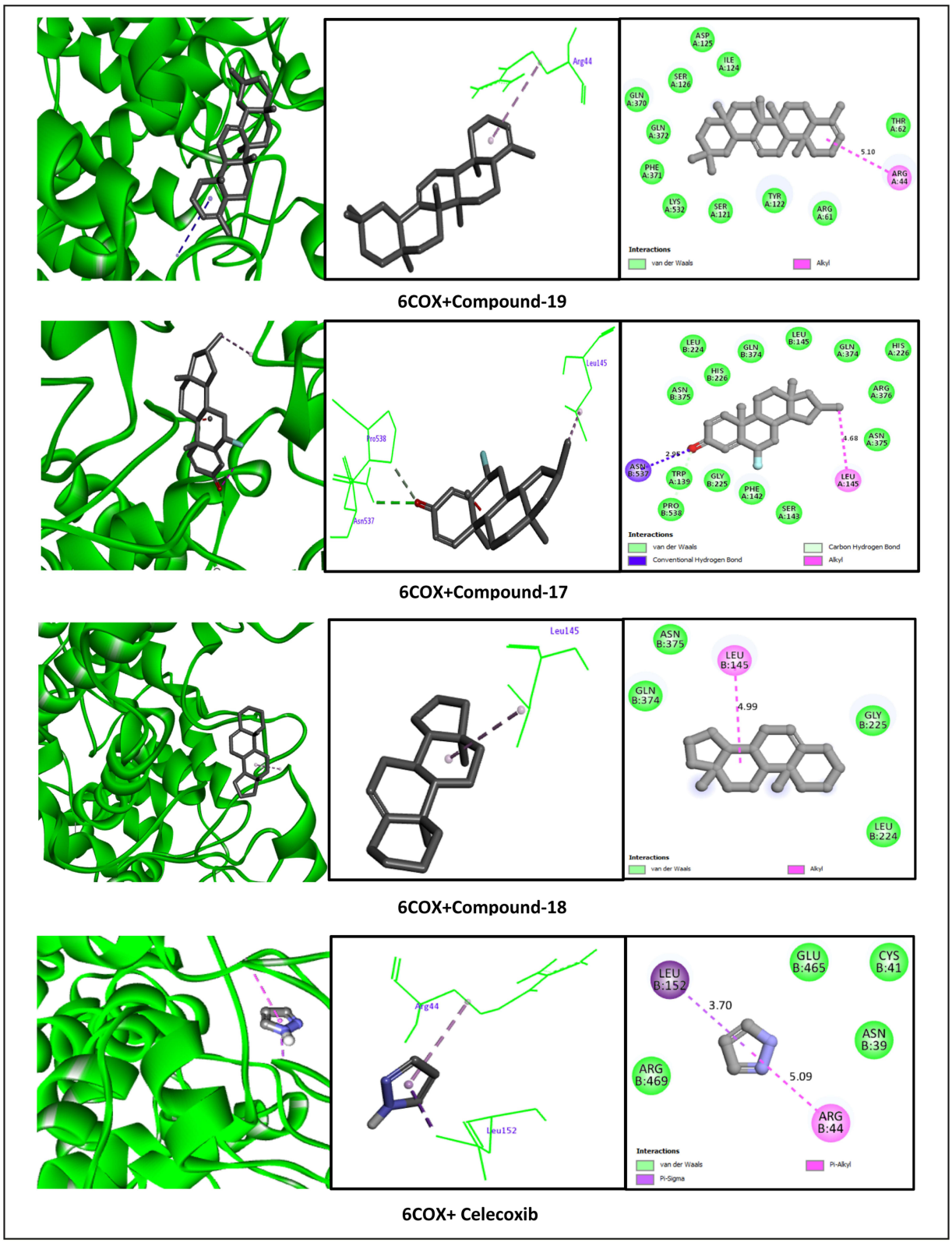


Figure 8 Graphical representation of the molecular interactions of the most prominent bioactive with the 6COX enzyme with 3D visualization.

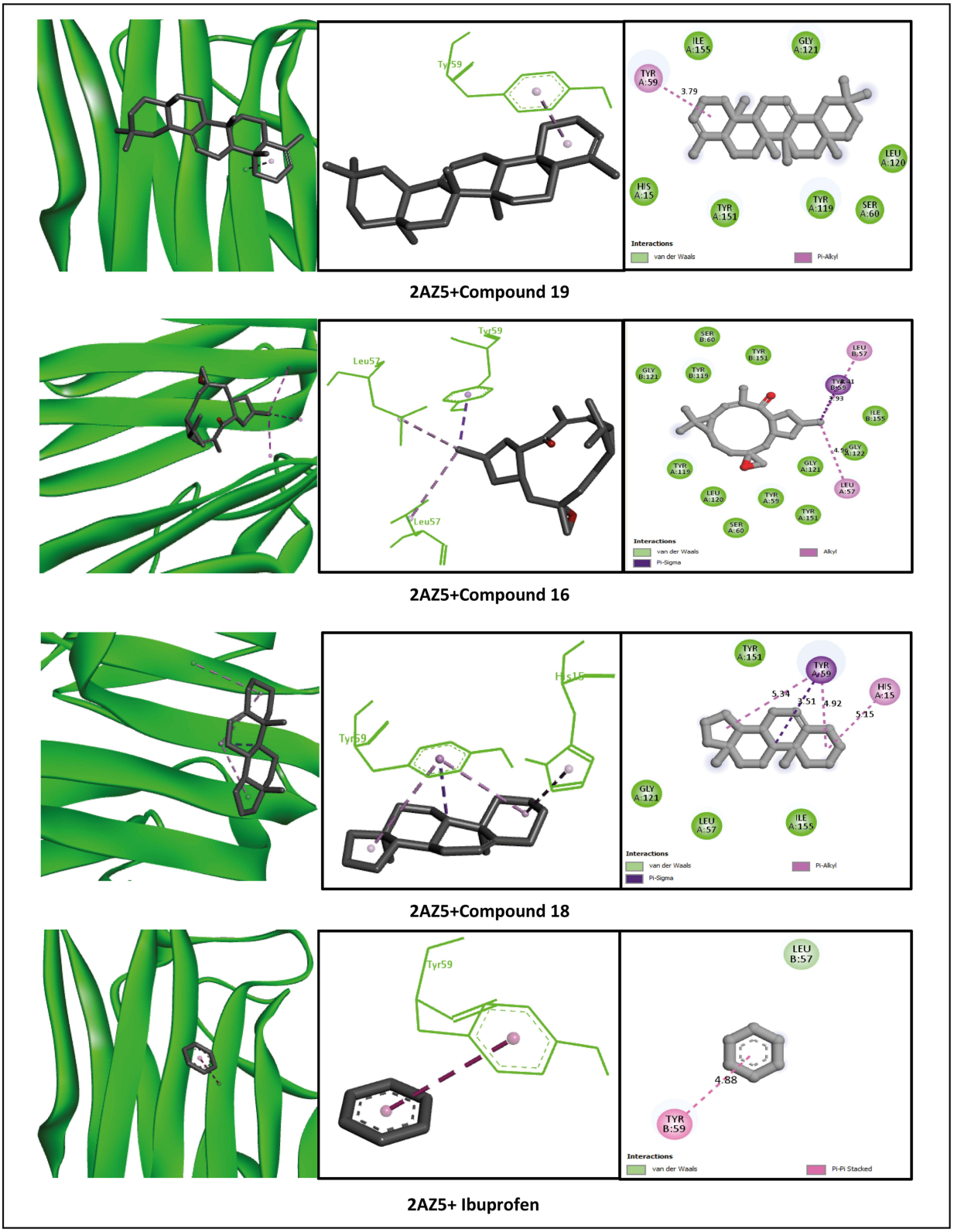


Figure 9 Graphical representation of the molecular interactions of the most prominent bioactive with the 2AZ5 enzyme with 3D visualization.

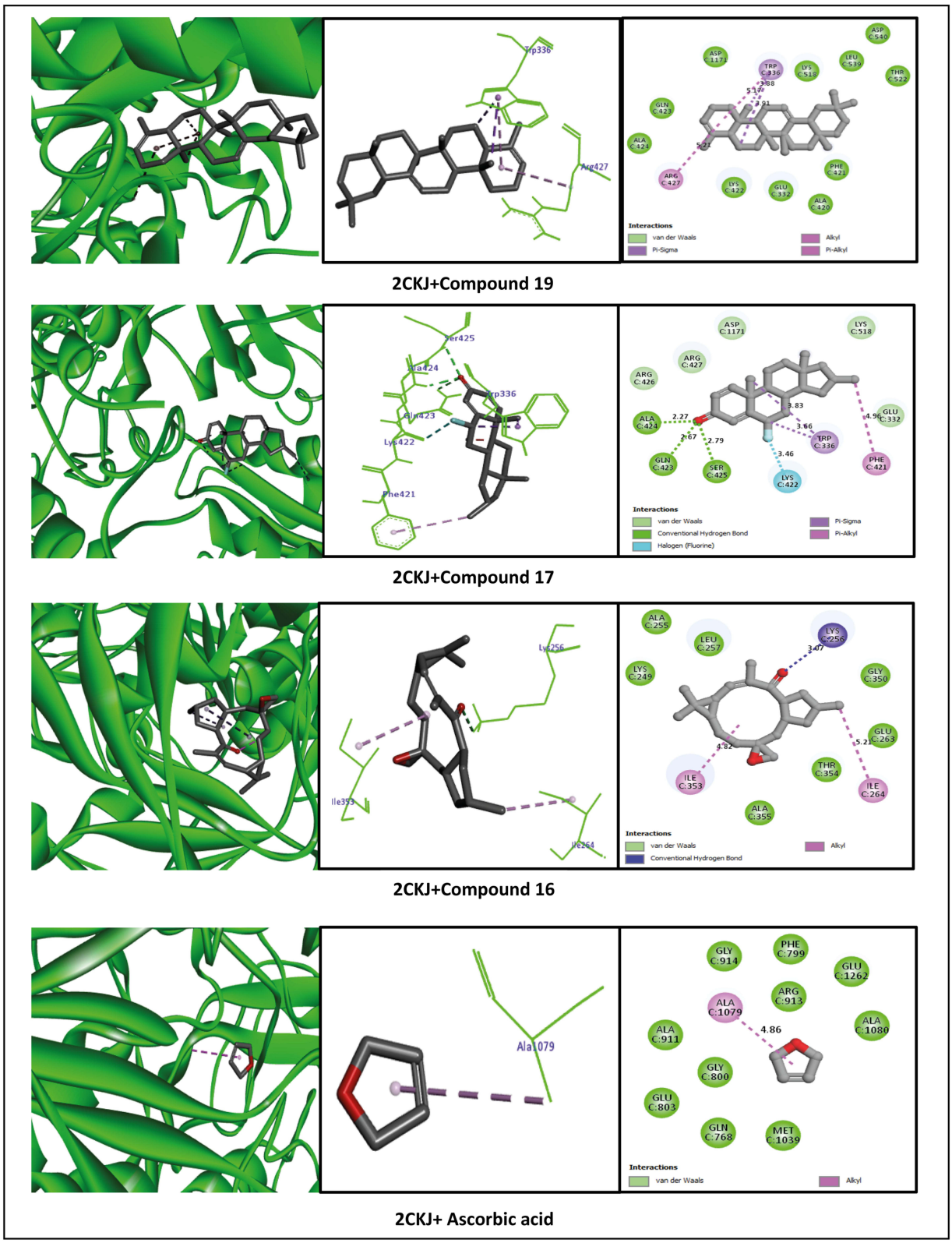


Figure 10 Graphical representation of the molecular interactions of the most prominent bioactive with the 2CKJ enzyme with 3D visualization.

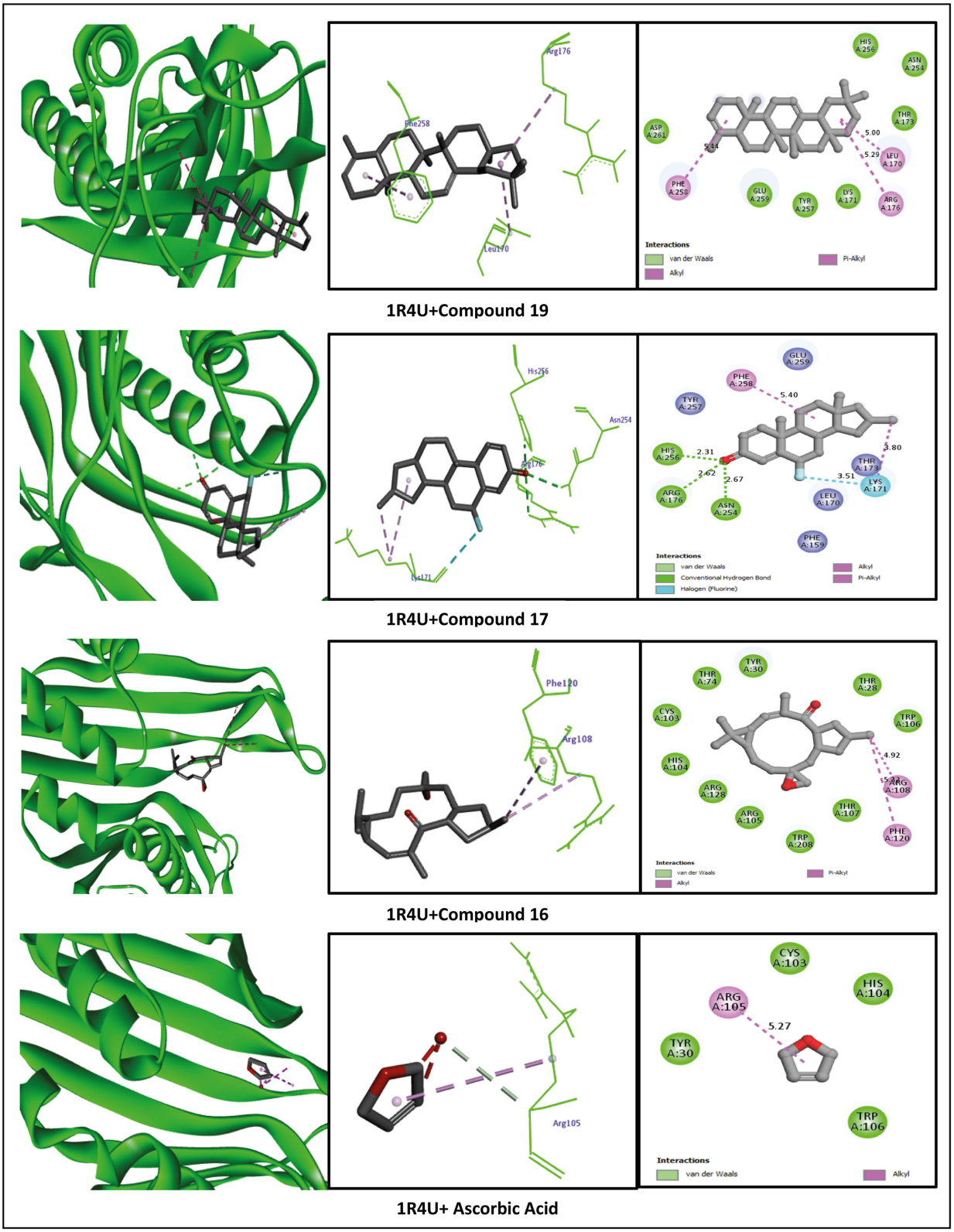


Figure 11 Graphical representation of the molecular interactions of the most prominent bioactive with the 1R4U enzyme with 3D visualization.

Table 13 ADME/T Properties Prediction of the Bioactive Compound of SIF-ME

SL no.	Compounds name	ADME							Toxicity					
		Lipinski rules				Lipinski violations ≤1	Verber rules		Parameters					
		MW (g/mol) <500	HBA <10	HBD <5	Log p ≤5		nRB ≤10	TPSA ≤140	Ames Toxicity	Carcinogen	Acute oral Toxicity	Human intestinal absorption	Human oral bio-availability	Blood-Brain Barrier
1	Butanoic acid, 3-methyl-	102.13	2	1	1.35	0	2	37.30	NAT	NC	III	0.9910	0.6857	0.9250
2	Phenol, 2-methoxy-	124.14	2	1	1.76	0	1	29.46	NAT	NC	II	0.9953	0.7571	0.5000
3	2-Methoxy-4-vinylphenol	150.18	2	1	2.14	0	2	29.46	NAT	NC	III	0.9956	0.5286	0.5750
4	Phenol, 2.6-dimethoxy-	154.16	3	1	1.85	0	2	38.69	NAT	NC	III	0.9923	0.6429	0.5250
5	Vanillin	152.15	3	1	1.57	0	2	46.53	NAT	NC	III	0.9961	0.5714	0.5500
6	Phenol, 2-methoxy-4-propyl-	166.22	2	1	2.46	0	3	29.46	NAT	NC	III	0.9948	0.5571	0.6000
7	Phenol, 4-ethenyl-2,6-dimethoxy-	180.20	3	1	2.28	0	3	38.69	NAT	NC	III	0.9931	0.5143	0.5250
8	Benzaldehyde, 3-hydroxy-4-methoxy-	152.15	3	1	1.44	0	2	46.53	NAT	NC	III	0.9961	0.6429	0.5500
9	5-Methyl-3-phenyl-1,3-oxazolidine	163.22	1	0	2.30	0	1	12.47	NAT	NC	III	0.9721	0.8429	0.9750
10	2,6-Dimethoxyhydroquinone	170.16	2	4	1.56	0	2	58.92	NAT	NC	III	0.9720	0.6000	0.5750
11	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	182.17	4	1	1.66	0	3	55.76	NAT	NC	III	0.9886	0.5571	0.5000
12	Phenol, 2.6-dimethoxy-4-(2-propenyl)-	194.23	3	1	2.46	0	4	38.59	NAT	NC	III	0.9919	0.5714	0.5500
13	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)	196.20	4	2	1.44	0	4	66.76	NAT	NC	III	0.9827	0.5571	0.6000

14	Retinoic acid	300.44	2	1	3.68	1	5	37.30	NAT	NC	III	0.9946	0.8143	0.8750
15	Octahydro-9-phenanthrene methanol	216.32	1	1	2.91	0	1	20.23	AT	NC	III	0.9921	0.5571	0.8750
16	Epoxylathyrol	350.45	5	3	2.84	0	0	90.29	NAT	NC	III	0.9751	0.5143	0.6000
17	Beta -Sitosterol	414.71	1	1	4.79	1	6	20.23	NAT	NC	III	0.9946	0.5714	0.9250
18	24-Noroleana-3,12-diene	394.68	0	0	4.86	1	0	0.00	NAT	NC	III	0.9910	0.6857	0.9250
Standards	Celecoxib	381.37	7	1	2.56	0	4	86.36	NAT	NC	III	0.9923	0.7857	0.9250
	Ibuprofen	206.28	2	1	2.17	0	4	37.30	NAT	NC	III	0.9947	0.9143	0.8750
	Ascorbic Acid	176.12	6	4	0.31	0	2	107.22	NAT	NC	IV	0.6225	0.5857	0.8750

Abbreviations: MW, Molecular weight; HBA, Hydrogen bond acceptor; HBD, Hydrogen bond donor; Log P, Lipophilicity; nRB, Number of rotatable bonds; TPSA, Topological polar surface area; AT, Ames toxicity; NAT, Not Ames Toxicity; NC, Non-Carcinogenic.

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prostaglandins by inhibiting the COX enzyme and preventing protein denaturation. Thus, the protein-denaturation test serves as a handy tool to examine its anti-inflammatory efficacy. SIF-ME exhibited the capability of reducing auto-antigen production, thereby preventing protein denaturation; it is comparable to diclofenac-Na.

The xylene-induced ear edema test facilitates the study of anti-inflammatory steroids and exhibits reduced responsive to NSAID agents.[57] Clinically, significant vasodilation, swelling alterations of the skin, and infiltration of inflammatory cells are found as symptoms of acute inflammation following topical administration of xylene.[58] In the current investigation, the increases in ear weight were inhibited in a dose-dependent way by the extract, suggesting a potential indication of the anti-phlogistic effects of the extract. Plants are rich in phenol and flavonoids and exhibit antioxidants by reducing oxidative stress.[59] Research suggests that the oxidation of flavonoids can lead to the formation of metabolites that retain or even enhance anti-oxidant properties, showcasing their ability to combat reactive oxygen species (ROS) effectively. Flavonoids, with their unique structural backbone and diverse subclasses, and essential for preventing anti-oxidant capacity and shielding against free radicals.[60] The previous study of Mahmud et al showed that the aqueous extract was effective in scavenging free radicals in the DPPH and ABTS tests, indicating vigorous antioxidant activity of *Shirakiopsis indica* fruits.[9] SIF-ME showed concentration-dependent antioxidant activity by inhibiting DPPH radical with an IC50 value of 469.5 µg/mL, and total phenol was determined to be 16.26 ± 0.67 of GAE/g, and flavonoid was determined to be 9.27 ± 0.20 mg/g of quercetin of crude extract. The findings revealed that SIF-ME exhibits a dose-dependent ability to donate hydrogen and is a significant source of antioxidants. Upon summarizing all outcomes, our conclusion indicated that alkaloids, phenols, flavonoids, terpenoids, tannins, ash, glycosides, and other compounds may be the primary contributors to the analgesic, anti-inflammatory, and anti-oxidant activity of SIF-ME.

Molecular docking study is commonly employed to study ligand-target interaction and understand the compound's biological activity by predicting their bindings.[61] Hence, molecular docking is conducted to align with the present analgesic, anti-inflammatory, and antioxidant findings and enhance comprehension of the molecular mechanism. Within this investigation, 18 compounds of SIF-ME that are not violet Lipinski and Veber's violation were examined against target receptors 5C1M (Mu-opioid) and 6COX (cyclooxygenase-2) for analgesic receptor, 2AZ5 (TNF-α) for anti-inflammatory receptor and 2CKJ (xanthine oxidoreductase), 1R4U (urate oxidase) for anti-oxidant receptor. Among the 18 compounds, every compound was found docked against receptors with a comparison to the standard drug. The best score against those receptors was shown that 24-Noroleana-3,12-diene exhibited both analgesic, anti-inflammatory, and antioxidant activity [4142]. Thus, the analgesic, anti-inflammatory, and antioxidant activity of SIF-ME can be described by the presence of 24-Noroleana-3,12-diene, Epoxylathyrol, Octahydro-9-phenanthrene methanol and Retinoic acid, which displayed excellent docking scores and also proved by GC-MS analysis.[40]

Screening of the analgesic, anti-inflammatory, and antioxidant activity of various bioactive compounds present in SIF-ME was executed utilizing the PASS program. Amongst the compounds, 24-Noroleana-3,12-diene showed the highest Pa values (0.726, 0.848, and 0.280) for analgesic, anti-inflammatory, and antioxidant activity.

Moreover, based on the top scores from molecular docking analysis, the bio-active compounds underwent ADME and toxicological assessments.[62] Adhering to Lipinski rule, drugs should have a molecular weight <500 amu, hydrogen bond acceptor <10, hydrogen bond donor <5, Log P ≤5, Number of rotatable bonds <10, and topological polar surface area (TPSA) ≤ 140Å for optimal oral bioavailability. Out of 60 compounds, 18 met all criteria, indicating robust bioavailability. However, 48 compounds violated Lipinski and Veber's rules, prompting toxicity evaluations. The toxicological study revealed minimal risk for Ames toxicity, carcinogenicity, and acute oral toxicity, except for octahydro-(-phenanthrene methanol. Consequently, most phytocomponds are promising drug candidates with favorable oral bioavailability.

New Insights and Novelty of the Research Study

Mangrove plants are reservoirs of diverse bioactive compounds, including alkaloids, flavonoids, and tannins, which exhibit a range of pharmacological activities. However, the exploration of these plants has been limited, and their full potential in drug discovery remains largely untapped. Increased research and attention to mangrove ecosystems could uncover new and effective therapeutic agents, offering valuable contributions to pharmaceutical science and medicine.[63] *Shirakiopsis indica* (Willd)., a distinguished mangrove plant, carries substantial ecological and medicinal significance.

Despite its potential, this plant remains underexplored, offering a promising avenue for future drug discovery and environmental conservation research.[64]

To the best of our knowledge, this research work marks a significant breakthrough in the first-timed chemical-biological exploration of the methanolic extract of *Shirakiopsis indica* (Willd). fruit. The novelty of this study is further underscored by the pioneering application of GC-MS/MS analysis to this plant, offering unprecedented insights into its chemical composition to project the prospective responsible bioactive phytochemicals.[65] This study also shed light on its hitherto uncovering new bioactivities of antioxidant-rich SIF-ME, including promising in vivo analgesic potential for the first time involved in the hot plate test, acetic acid-induced writhing test, and formalin-induced licking test. Besides, anti-inflammatory efficacy through the human RBC membrane stabilization assay (HRBC), protein denaturation assay, and xylene-induced ear edema methods provide essential clues for further drug discovery research. Moreover, corresponding in silico studies also give essential hints on prospective bioactive secondary metabolites.

Limitations of the Study

This study addressed some of the in vivo and in vitro biological potentials along with the identification of sixty (60) bioactive secondary metabolites from GC-MS/MS analysis of *Shirakiopsis indica* (Willd). fruit extract. However, for the discovery and optimization of the lead compounds responsible for the reported biological activities of the extract, relevant secondary metabolites must be isolated, characterized, and further analyzed to determine the relevant mode of action, safety, efficacy, and dosing profiles, followed by clinical trials.[66,67] Besides, Pk/Pd profiling, bioavailability, and toxicological parameters investigations of plant extract are also required for the drug development process. In this regard, our study will act as a linkage and a breakthrough in future studies, as it provides a conspicuous hint for probably responsible lead discovery via identifying an array of phytochemicals by GC-MS/MS and gives insights on some of the substantial bioactivities (in vivo and in vitro) supported by respective in silico studies. Such investigations will pave the way for future studies to harness its potential in various applications.

Conclusion

As per our research findings, the SIF-ME has shown considerable potential in every facet of herbal medicine. Our research has demonstrated that the plant is abundant in bioactive phytochemicals showing analgesic, anti-inflammatory and anti-oxidant action by in-vitro, in-vivo, and in-silico experiments.

In our study, we found that 24-Noroleana-3,12-diene may have the potential to function in the treatment of pain, inflammation, and anti-oxidants. However, additional comprehensive investigations are required to evaluate the effectiveness of this chemical as an analgesic, anti-inflammatory, and antioxidant molecule. Further study is essential to grasp the therapeutic capability of these substances comprehensively and offer secure and efficient treatment options for patients.

Ethical Approval

This study was approved by the institutional review board of the University of Science & Technology, Chittagong, Bangladesh (No. USTC/EAC/24/020) on animal experimentations and human samples tests. The human studies and animals were in accordance with the principle of the Helsinki Declaration. All the participants provided their informed consent. Consent was received from patients to report individual data. Animal models were handled and treated according to the principle of the Swiss Academy of Science and were euthanized following the Guidelines for the Euthanasia of Animals.

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Disclosure

The authors declare no conflicts of interest in this work.

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