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RESEARCH ARTICLE

Evaluation of anti-nociceptive and anti-inflammatory activities of *Piper sylvaticum* (Roxb.) stem by experimental and computational approaches

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Abstract

Piper sylvaticum Roxb., (Family: Piperaceae), commonly known as pahaari peepal, is used in traditional medicine for the treatment of rheumatic pain, headache, asthma, chronic cough, diarrhea, and wounds. To provide scientific proof for its traditional use, the present study was designed to investigate the antinociceptive and anti-inflammatory properties of methanol extract of *P. sylvaticum* stem (MEPSS) in pain models. Additionally, computational studies viz. molecular docking, ADME and toxicological property predictions were performed to identify the potent phytochemicals of this plant for antinociceptive and anti-inflammatory activities with good oral bioavailability and safety features. Quantitative phytochemical analysis of MEPSS was performed using established protocols. The antinociceptive activity was determined using acetic acid and formalin test in mice at the doses of 200 and 400 mg/kg while paw edema induced by carrageenan used for anti-inflammatory activity. Molecular docking study was performed by Schrödinger Maestro 10.1 whereas the SwissADME and admetSAR were used for ADME and toxicity prediction respectively. The total phenolic and flavonoid contents of MEPSS were 93.39 and 53.74 mg gallic acid and quercetin equivalent/g of extract respectively. The methanol extract exhibited significant and dose-dependent antinociceptive and anti-inflammatory effects in experimental pain models. Also, our docking study showed that piperine, piperlonguminine, and sylvamide have the best binding affinities to cyclooxygenase enzymes with good ADME/T properties. This study confirmed that MEPSS possess significant antinociceptive and anti-inflammatory activities which could be due to the presence of phytochemicals and three bioactive compounds (piperine, piperlonguminine, and sylvamide) were found to be most effective in computational studies.

Keywords *Piper sylvaticum* · Antinociceptive · Anti-inflammatory · Molecular docking · ADME and toxicity prediction

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Abbreviations

MEPSS	Methanol extract of *Piper sylvaticum* stem
ADME	Absorption, distribution, metabolism, elimination
p.o	Per oral
OECD	Organization for Economic Co-operation and Development
PDB	Protein data bank
OPLS	Optimized potentials for liquid simulations
RMSD	Root-mean-square deviation
SPSS	Statistical package for the social sciences
SEM	Standard error of the mean
ANOVA	Analysis of variance

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Introduction

Pain acts like an alarm that enables us to analyze any possible threats or hazard but when it results in pathological condition then it is important to control the pain. The different forms of pathological situations such as chronic, limb, cancer pains are challenging regarding the complexity but however important to control (Cortes-Altamirano et al. 2018). Such problems negatively impact the quality of the life of patients and provide further evidence for the need for delivering a robust solution as novel therapeutic targets for pain management. The study using mouse models suggest that chronic pain instigate dysfunction in the noradrenergic transmission in the locus coeruleus in the neuropathic pain (Ide et al. 2015). Previously studied research has reported the use of natural crude extracts from *A. crucigerum* seeds (De Prá et al. 2017), opioids (Ide et al. 2015), crude methanolic extracts (Paul et al. 2018), paracetamol (Roca-Vinardell et al. 2018), *Muntingia calabura* leaves extract (Zakaria et al. 2016), *Bauhinia glauca* (Xu et al. 2015) phytochemical constituent's revealed the antinociceptive effects in pain. Regardless of advances that have been made in the domain of pain therapy, it is vital to find new analgesic agents with the significant effect that have less or no side effects for the treatment of several painful chronic diseases.

The genus piper is a wide variety of commercially available medicinal plants that belong to the Piperaceae family. It can be easily found in tropical and subtropical countries such as Bangladesh, India, China, Malaysia, Indonesia, and Myanmar. The Piperaceae family of the plant kingdom is further sub grouped to 13 genera which currently contains more than 3600 species (Parmar et al. 1997). The plant belonging to this group has been commonly known for high medicinal values, this is both effective and efficient in stomach pain, chest pain and acting as anti-inflammatory agents (Kumar et al. 2016). The chemistry and chemical constituents of Piper species have been studied well and have led to the isolation of many chemically active compounds like flavones, lignans, amide alkaloids, terpenes, chalcones, etc. (Parmar et al. 1997). One of the less studied species from piper group is *Piper sylvaticum* (Roxb.) also known as *Chavica sylvatica* (Roxb.) are climber's herbaceous, habitation in wet places. It is locally known as pahari pipul (Hindi), pahaari peepal (Folk), vana-pippali (Ayurveda), and mountain long pepper (English). This plant has a wide range of therapeutic applications in the practice of traditional medicine in asthma, chronic cough, cold, piles, diarrhea, rheumatic pain, headache, tuberculosis, wounds, indigestion, and dyspepsia (Quattrocchi 2012; Sai ePublications 2013). A preliminary phytochemical study showed that it contains alkaloids, flavonoids, carbohydrates, saponins, and tannins (Paul et al. 2018). Additionally, six major compounds have also been isolated from this plant such as piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone (Parmar et al. 1997). Previously reported data regarding pharmacological studies of this plant revealed that the plant possesses antioxidant (Kumar et al. 2016) and anthelmintic (Paul et al. 2018) properties. Several courses of action of *Piper sylvaticum* has been already investigated in the literature, however, there has been no study about the potential use as an antinociceptive and anti-inflammatory activity. As a result, this set of studies with the use of experimental and computational approaches was performed to investigate the antinociceptive and anti-inflammatory activities of *Piper sylvaticum* (Roxb.) stem for the very first time.

Materials and methods

Drugs and chemicals

Methanol, potassium acetate, formalin, acetic acid, and sodium carbonate were purchased from Merck (Darmstadt, Germany) while quercetin and Tween-80 from BDH Chemicals Ltd. Gallic acid, Folin–Ciocalteau reagent, and aluminum chloride were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Diclofenac sodium was obtained from Square Pharmaceuticals Ltd., Bangladesh and absorbance was measured by using UV–Vis spectrophotometer (UVmini-1240, Shimadzu, Japan).

Plant material collection and identification

The stems of *Piper sylvaticum* (Roxb.) was collected from Sita Pahar area of Kaptai, Chittagong, Bangladesh in October 2014 and the collected plant was authenticated by Dr. Shaikh Bokhtear Uddin, botanist, from University of Chittagong with a reference number (SUB 3217) which has been deposited in the Herbarium of the University of Chittagong for future reference.

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Preparation of extract

Approximately 220 g of the powdered materials were soaked in 700 ml of methanol at room temperature for 14 days with occasional stirring and shaking. The resultant mixture was filtered through a cotton plug followed by Whatman No.1 filter paper and the filtrate solution evaporated to yield the methanol extract of *P. sylvaticum* stem. The extract showed a yield of 4.54% and stored in a refrigerator at 4 °C for further analysis.

Experimental animals

Swiss albino mice of both sexes (weighing about, 20–25 g) were collected from Jahangir Nagar University, Savar, Dhaka, Bangladesh. The animals were sheltered in polypropylene cages by maintaining suitable laboratory conditions (room temperature 25 ± 2 °C; relative humidity 55–60%; 12 h light/dark cycle) along with standard laboratory food and distilled water ad libitum. All the experimental works were conducted in a noiseless condition and the animals were acclimatized to laboratory conditions for 10 days before experimentation.

Ethical statement

This study was carried out in accordance with the internationally accepted principle for proper use of laboratory animals namely National Institutes of Health (NIH) and the International Council for Laboratory Animal Science (ICLAS). The present study protocol was reviewed and approved by the "P&D committee" of the Department of Pharmacy, International Islamic University Chittagong, Bangladesh with a reference number: Pharm-P&D-61/08'16-125.

Determination of total phenolic and flavonoid content

The total phenolic content (Harborne 1998) was determined using Folin-Ciocalteu method and expressed as milligrams of gallic acid equivalents (mg GAE/g dried extract), whereas total flavonoid content (Aiyegoro and Okoh 2010) was determined by $AlCl_3$ assay and expressed as milligrams of quercetin equivalents (mg QE/g dried extract). The experiment was conducted in triplicates, and the results were expressed as mean ± SEM.

Acute oral toxicity study

Based on the OECD guidelines (No 2001), acute oral toxicity study of MEPSS was carried out using the limit dose of 2000 mg/kg, body weight of the mice.

Antinociceptive activity

Dosing groups

In the present study, mice were randomly divided into four groups and each group consisting of six mice (n = 6). Here, the control group received 1% Tween-80 in distill water, the positive control group received reference standard drug, Diclofenac sodium (10 mg/kg, body weight) whereas the remaining groups were given 200 and 400 mg/kg, body weight of MEPSS.

Acetic acid-induced writhing test in mice

The acetic acid-induced writhing test was performed based on the previously reported method (Koster et al. 1959). Thirty minutes after the administration of doses, 0.6% acetic acid (10 ml/kg body weight) was injected intraperitoneally (i.p) into the mice. After 5 min of acetic acid injection, the number of writhing (abdominal constrictions) was counted for 15 min and the writhing responses were compared with the control group. Antinociceptive activity was expressed as a percentage of writhing inhibition and calculated using the following formula: percent of inhibition (%) = [(Control group − Test groups)/Control group] × 100.

Formalin-induced licking test in mice

The formalin-induced paw licking test was performed based on the previously described method (Hunskaar and Hole 1987). Thirty minutes after the administration of doses, 20 µl of 2.5% formalin was injected subcutaneously (s.c) into the sub-plantar region of the right hind paw of mice. In this test, pain response was reflected as indicative of the nociceptive behavior and the total time spent in the behavioral responses to nociception including licking/biting of the injected paw was noted. The entire time spent was noted up to 30 min where the first 5 min (0–5 min) was considered as an early phase or neurogenic phase and last 15 min (15–30 min) considered as a late phase or inflammatory phase. The percentage of inhibition of antinociceptive activity was calculated as described in the previous method.

Anti-inflammatory activity

Carrageenan-induced paw edema test in mice

The anti-inflammatory activity of MEPSS was performed by the induction of carrageenan into the plantar surface of the mice hind paw based on the previously reported protocol (Lanhers et al. 1991). The animals were divided into four groups (n = 6) where Group I (control) received 1% Tween-80 (2 ml/kg) and Group II (reference standard drug)

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received Diclofenac sodium (10 mg/kg body weight; p.o); Group III and IV received 200 and 400 mg/kg body weight of the MEPSS per oral respectively. A suspension of 0.05 ml carrageenan (1 mg/kg) dissolved in 1% Tween-80 with distill water was injected in the subplantar area of the right paw of the mice to induce acute inflammation of all four groups, and micrometer slide calipers were used to measure the paw volume at 1, 2, 3, and 4 h after test doses administration. The percentage inhibition of the inflammatory effect of the extract was calculated using the following equation: % inhibition of inflammation = [(Mean degree of inflammation (control–test groups))/Mean degree of inflammation of control] × 100.

Selection of compounds for the computational study

Piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone were selected based on the availability as major compounds through literature review (Parmar et al. 1997) and the chemical structures of the compounds were downloaded from the PubChem database.

Chemical compounds studied in this article

Piperine (PubChem CID: 638024); piperlonguminine (PubChem CID: 5320621); sylvamide (PubChem CID: 21580215); sylvatine (PubChem CID: 90472536); sylvatesmin (PubChem CID: 3083590); and sylvone (PubChem CID: 15043005).

Molecular docking analysis

Ligand preparation

The chemical structures of six major representative compounds were obtained from PubChem compound repository (Fig. 1), neutralized at pH 7.0 ± 2.0 and minimized by LigPrep tool (force field OPLS_2005) embedded in Schrödinger suite-Maestro v 10.1.

Enzyme preparation

Three-dimensional crystallographic enzyme structures were downloaded from the Protein Data Bank RCSB PDB (Berman et al. 2002): cyclooxygenase-1 (COX-1, PDB id: 2OYE) (Harman et al. 2007) and cyclooxygenase-2 (COX-2, PDB id: 3HS5) (Vecchio et al. 2010). The enzyme was prepared for docking experiment by Protein Preparation Wizard embedded in Schrödinger suite-Maestro v 10.1 as we previously described (Hasanat et al. 2017).

Glide docking

Molecular docking studies were performed to elucidate the possible mechanism of the selected compounds against COX enzymes for antinociceptive and anti-inflammatory activity. Docking experiments were carried out using Glide embedded in Maestro by standard precision scoring function as we previously described (Hasanat et al. 2017).

Predictions of pharmacokinetic parameters by SwissADME

The pharmacokinetic properties of the compounds were predicted by using SwissADME online server (http://www.swissadme.ch/). In the current study, molecular descriptors such as molecular weight, Hydrogen bond acceptor, Hydrogen bond donor, Lipophilicity, molar refractivity, number of rotatable bond, topological polar surface area, Percentage of absorption and violations of Lipinski's rule of five were calculated since orally active drugs should comply with these widely utilized drug-likeness properties to ascertain their pharmaceutical credibility.

Toxicological properties prediction by admetSAR

Toxicological properties of the compounds were determined by the admetSAR online server (http://lmmd.ecust.edu.cn/admetsar1/predict/) as toxicity is a prime issue during the drug discovery process. In this study, Ames toxicity, carcinogenic properties, acute oral toxicity, and rat acute toxicity were predicted.

Statistical analysis

Results were expressed as mean ± SEM whereas SPSS software (version 20) was used for statistical analysis and all comparisons were made by using one-way ANOVA followed by Dunnett's test. A p value less than 0.001 considered as statistically significant.

Results

Total phenol and flavonoid contents

The result of total phenolic and flavonoid contents are shown in Table 1. The total phenol and flavonoid contents of MEPSS were 93.39 ± 2.88 mg GAE/g and 53.74 ± 0.39 mg QE/g dried extract, respectively.

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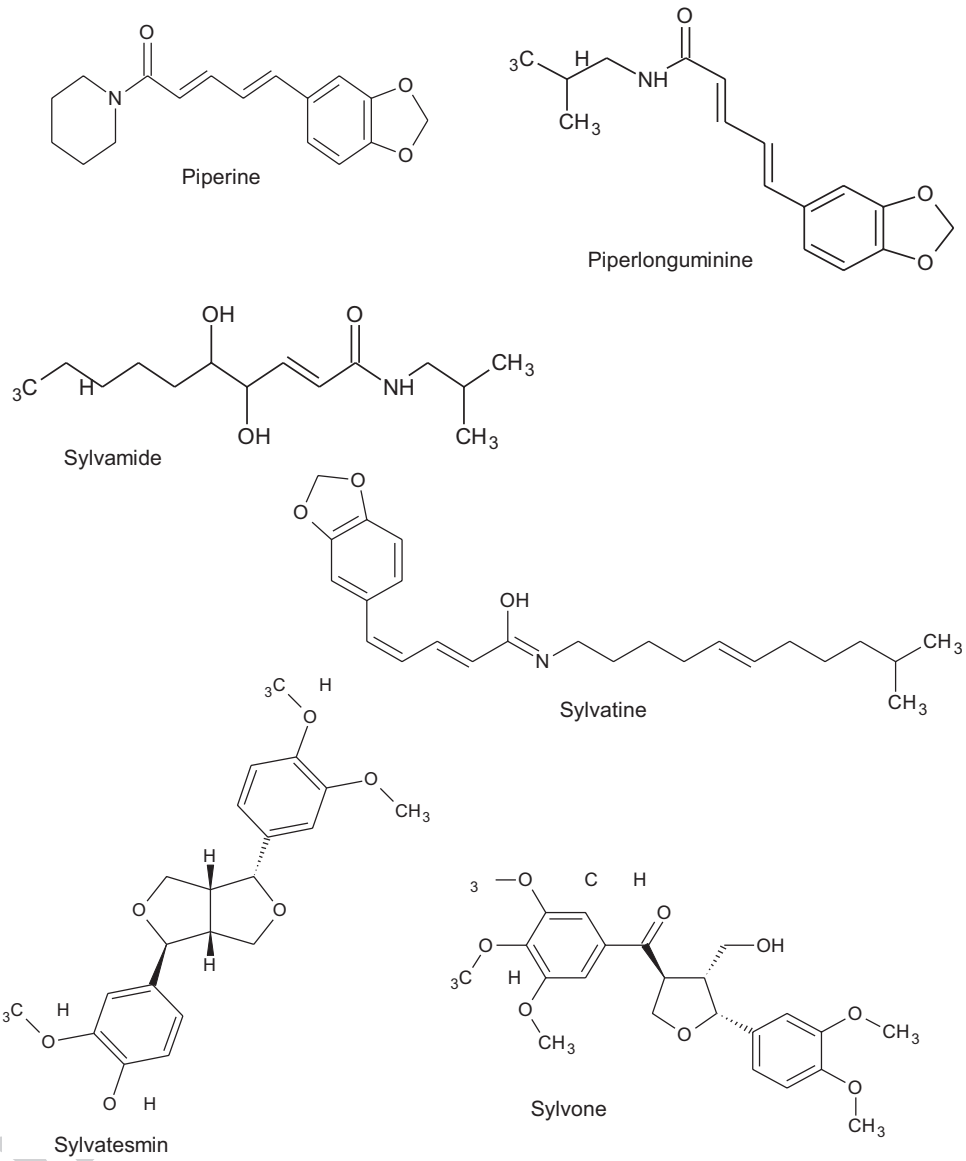

Fig. 1 Chemical structures of compounds piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone used for computational study

Table 1 Total phenol and flavonoid contents of methanol extract of *Piper sylvaticum* stem

Tested extract	Total phenol content (mg GAE/g dried extract)	Total flavonoid content (mg QE/g dried extract)
MEPSS	93.39 ± 2.88	53.74 ± 0.39

Each value is expressed as mean ± SEM (n = 3)

MEPSS methanol extract of *Piper sylvaticum* stem, *GAE* gallic acid equivalent, *QE* quercetin equivalent

Acute toxicity test

No mortality, behavioral and neurological changes were observed at the specified doses during the 72 h of the observation period. Therefore, the dose up to 2000 mg/kg was considered as safe for MEPSS.

Antinociceptive activity

Acetic acid test

In the acetic acid-induced writhing test, the methanol extract exhibited 37.17 and 59.95% ($p < 0.001$) inhibition of writhing at the doses of 200 and 400 mg/kg body weight, respectively, where the reference standard drug (diclofenac sodium, 10 mg/kg body weight) showed 70.74% inhibition as compared to the control group, and the results were statistically significant (Fig. 2).

Formalin test

The result of MEPSS on formalin test is given in Table 2. In this test, the methanol extract showed a dose-dependent

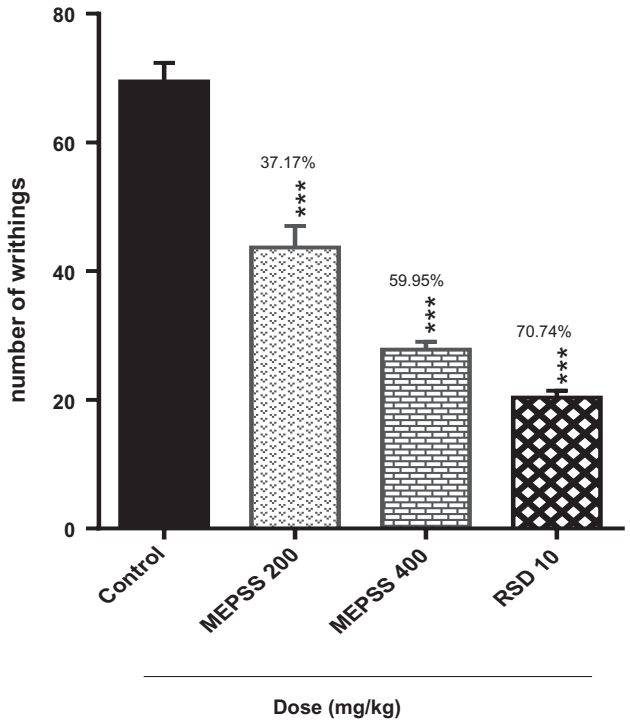

Fig. 2 Antinociceptive effect of methanol extract of *Piper sylvaticum* stem (MEPSS) in acetic acid induced writhing test. Results are expressed as mean ± SEM (n = 6); RSD, Reference standard drug (Diclofenac sodium). ***$p < 0.001$ compared with the control group (Dunnett's Test)

antinociceptive effect by decreasing the paw licking time in both the early and late phases. Oral administration of the extract at the doses of 200 and 400 mg/kg body weight, significantly reduced the paw licking time by 38.66 and 47.85% ($p < 0.001$) respectively, in the early phase and by 30.79 and 41.51% ($p < 0.001$) respectively, in the late phase of the test. The reference standard drug (Diclofenac sodium, 10 mg/kg body weight) significantly reduced formalin-induced nociception in the early and late phases 63.97 and 71.32% ($p < 0.001$) respectively.

Anti-inflammatory activity by carrageenan-induced paw edema test

The anti-inflammatory effect of MEPSS using carrageenan-induced paw edema test is shown in Table 3. Swiss albino mice treated with an oral dose of 400 mg/kg MEPSS showed statistically significant 32.97% ($p < 0.01$), 41.66% ($p < 0.001$), 49.23% ($p < 0.001$) and 58.18% ($p < 0.001$) reduction in paw edema at 1, 2, 3, and 4 h, respectively. The reference standard drug (Diclofenac sodium, 10 mg/kg) also showed statistically ($p < 0.001$) significant 32.97%, 45.23%, 52.30% and 70.90% reduction at 1, 2, 3, and 4 h time points, respectively when compared to control group.

Molecular docking study

In the current study, six major compounds of *P. sylvaticum* were docked against COX-1 (PDB: 2OYE) and COX-2 (PDB: 3HS5) enzymes. Our result showed that piperlonguminine has the best binding affinity against COX-1 enzyme with the highest docking score −4.95 kcal/mol followed by piperine (−4.91 kcal/mol), sylvamide (−3.27 kcal/mol) and sylvatine (−1.51 kcal/mol). However, two compounds specifically sylvatesmin and sylvone didn't bind with the COX-1 enzyme. On the other hand, piperine (−8.88 kcal/mol) was found to have the highest binding affinity to the COX-2 enzyme with the highest docking score followed by piperlonguminine (−6.40 kcal/mol), and sylvamide (−1.62 kcal/mol). But, three compounds viz. sylvatine, sylvatesmin and sylvone didn't dock with COX-2 enzyme. The result of the docking study is shown in Table 4 and the docking figure is presented in Figs. 3, 4, 5 and 6.

Docking analysis of each compound suggested several binding interactions between the ligands and the target enzymes. Piperine interacts with COX-1 enzyme through one hydrogen bond to Ser87 and three hydrophobic interactions with His95 (two interactions) and Pro514 (docking score -4.91 kcal/mol). Piperlonguminine interacts with the same enzyme through the formation of two hydrogen bonds with Gly354 and His513 residues, and three hydrophobic interactions with His513 (two interactions) and Pro514

Table 2 Antinociceptive effect of methanol extract of *Piper sylvaticum* stem in formalin induced paw licking test in mice

Samples	Dose (mg/kg)	Licking time (s) (Mean ± SEM)			
		Early phase (0–5 min)	Inhibition (%)	Late phase (15–30 min)	Inhibition (%)
Control	0.1 ml/mouse	57.27 ± 1.23	–	41.61 ± 1.11	–
RSD	10	20.63 ± 0.82***	63.97	11.93 ± 1.08***	71.32
MEPSS	200	35.13 ± 0.72***	38.66	28.79 ± 0.90***	30.79
MEPSS	400	29.86 ± 1.33***	47.85	24.34 ± 0.65***	41.51

Each value in the table is represented as mean ± SEM (n = 6)

MEPSS methanol extract of *Piper sylvaticum* stem, *RSD* reference standard drug (Diclofenac sodium)

***$p < 0.001$ compared with the control group (Dunnett's Test)

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Table 3 Anti-inflammatory activity of methanol extract of *Piper sylvaticum* stem on carrageenan induced paw edema test in mice

Treatment (mg/kg)	Paw volume (mm) (% inhibition)			
	$T_{1\,h}$	$T_{2\,h}$	$T_{3\,h}$	$T_{4\,h}$
Control	0.410 ± 0.011	0.390 ± 0.008	0.352 ± 0.004	0.332 ± 0.008
RSD 10	0.340 ± 0.010*** (32.97)	0.306 ± 0.007*** (45.23)	0.276 ± 0.007*** (52.30)	0.246 ± 0.009*** (70.90)
MEPSS 200	0.378 ± 0.006 (23.40)	0.354 ± 0.004* (28.57)	0.322 ± 0.006* (32.30)	0.302 ± 0.003* (38.18)
MEPSS 400	0.354 ± 0.009** (32.97)	0.326 ± 0.012*** (41.66)	0.294 ± 0.009*** (49.23)	0.274 ± 0.015*** (58.18)

Each value is expressed as mean ± SEM (n = 6)

MEPSS methanol extract of *Piper sylvaticum* stem, *RSD* reference standard drug (Diclofenac sodium)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control group (Dunnett's test)

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(docking score − 4.95 kcal/mol). Sylvamide interacted through seven hydrogen bonds with Ser516, Pro514 (2 interactions), Gly354, Thr94, His90, and Gly354 (docking score − 3.27 kcal/mol) whereas Sylvatine interacted through two hydrophobic interactions with His90 (two interactions) with a docking score of − 1.51 kcal/mol. Though, sylvatesmin and sylvone did not show any interactions.

Oppositely, Piperine binds to the COX-2 enzyme by forming one hydrogen bond with Edo620 (docking score − 8.88 kcal/mol) five hydrophobic interactions with Leu352, Val116, Val349, Leu359, and Ala527. Piperlonguminine (docking score − 6.40 kcal/mol) interacted with the same enzymatic by forming one hydrogen bond with Leu531 and eight hydrophobic interactions with Leu352, Phe381, Tyr385 (two interactions), Tyr387, Val349, Ala527, and Leu531. Sylvamide (docking score − 1.62 kcal/mol) interacted through three hydrogen bonds with Met522, Ser353, and Edo620 and seven hydrophobic interactions with Ala527, Val349, Leu359, Val523, Tyr355, Phe381, and Tyr385. However, sylvatine, sylvatesmin, and sylvone did not show any interactions.

Pharmacokinetic and toxicological properties

According to Lipinski's rule of five, a compound could show drug-likeliness properties if it does not fail more than one of the following principles: (i) molecular weight not more than 500; (ii) H-bond acceptors ≤ 10; (iii) H-bond donors ≤ 5; (iv) Lipophilicity < 5; and (v) molar refractivity between 40 and 130. In this study, all of the compounds satisfied the Lipinski's rule of five and Veber's rule which clearly indicates all three compounds could be a suitable candidate for the new drug development process (Table 5).

On the other hand, toxicological parameters were predicted by admetSAR online server and results are shown in Table 6. Here, all the compounds showed non-ames toxic and non-carcinogenic properties. Additionally, all of the compounds showed weak rat acute toxicity with an LD_{50} (median lethal dose) value of 1.7848 to 2.7129 mol/kg while acute oral toxicity values lies in "class III" that means compounds of this class have LD_{50} values greater than 500 mg/kg but less than 5000 mg/kg and are suitable candidate for new drug.

Discussion

Pain is a serious global health problem as it affects all populations, regardless of age, sex, income, race or ethnicity, or geography. Moreover, it has a serious consequence like depression, inability to work, disrupted social relationships and suicidal thoughts. Globally, it has been estimated that one in five adults (20%) suffers from pain and that another one in ten adults (10%) are newly diagnosed with chronic pain each year. It reduces not only quality of life but also has a high impact on health and economy (Goldberg and Mcgee 2011; Adnan et al. 2019a, b). From this view, many experimental studies have been performed nowadays to explore new therapeutic agents of pain. Through the progress has been made in the development of new pain therapies, it is still necessary to find out new analgesic agents with fewer side effects for the treatment of various painful conditions. In this study, we examined the antinociceptive and anti-inflammatory activities of the methanol extract of *P. sylvaticum* stem in different pain models followed by computational analysis (In silico molecular docking, ADME and toxicity predictions).

For the experimental evaluation of the antinociceptive activity of MEPSS, we started our investigation with the acetic acid-induced writhing or abdominal constriction test in mice since this test is commonly used for the assessment of central and peripheral antinociceptive actions of new compounds (Ikeda et al. 2001). Intraperitoneal injection of acetic acid on the hind paw of mice initiates the release of endogenous prostaglandins (PGs), histamine, bradykinin, serotonin, cyclooxygenase (COX), lipooxygenase (LOX)

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Table 4 Docking scores and binding interactions of the selected compounds against COX-1 and COX-2 enzymes

Compounds	Docking score (kcal/mol)	Hydrogen bond interactions		Hydrophobic interactions	
		Amino acid residue	Distance (Å)	Amino acid residue (bond)	Distance (Å)
COX-1 (PDB: 2OYE)					
Piperine	−4.91	Ser87	2.87	His95 (Pi-Sigma)	2.68
				His95 (Pi–Pi T-shaped)	4.84
				Pro514 (Pi-Alkyl)	4.76
Piperlonguminine	−4.95	Gly354	2.44	Pro514 (Alkyl)	4.50
		His513	3.06	His513 (Pi-Alkyl)	4.53
				His513 (Pi-Alkyl)	5.44
Sylvamide	−3.27	Ser516	1.86	Phe356 (Pi-Alkyl)	5.46
		Pro514	1.75	His513 (Pi-Alkyl)	4.29
		Pro514	1.57	–	–
		Gly354	2.39	–	–
		Thr94	2.58	–	–
		His90	2.07	–	–
		Gly354	2.27	–	–
Sylvatine	−1.51	–	–	His90 (Pi-Alkyl)	4.99
				His90 (Pi-Alkyl)	4.46
Sylvatesmin	–	–	–	–	–
Sylvone	–	–	–	–	–
COX-2 (PDB: 3HS5)					
Piperine	−8.88	Edo620	3.02	Leu352 (Alkyl)	5.11
				Val116 (Pi-Alkyl)	4.73
				Val349 (Pi-Alkyl)	5.24
				Leu359 (Pi-Alkyl)	4.91
				Ala527 (Pi-Alkyl)	5.09
Piperlonguminine	−6.40	Leu531	2.41	Leu352 (Alkyl)	5.07
				Phe381 (Pi-Alkyl)	5.24
				Tyr385 (Pi-Alkyl)	5.42
				Tyr385 (Pi-Alkyl)	4.38
				Tyr387 (Pi-Alkyl)	4.34
				Val349 (Pi-Alkyl)	4.75
				Ala527 (Pi-Alkyl)	4.44
				Leu531 (Pi-Alkyl)	5.38
Sylvamide	−1.62	Met522	2.01	Ala527 (Alkyl)	4.21
		Ser353	2.45	Val349 (Alkyl)	4.06
		Edo620	2.26	Leu359 (Alkyl)	5.40
				Val523 (Alkyl)	5.17
				Tyr355 (Pi-Alkyl)	4.57
				Phe381 (Pi-Alkyl)	4.55
				Tyr385 (Pi-Alkyl)	4.45
Sylvatine	–	–	–	–	–
Sylvatesmin	–	–	–	–	–
Sylvone	–	–	–	–	–

and cytokines like IL-8, IL-1β, and TNF-α in the peripheral fluid tissue. These endogenous inflammatory mediators then enter into the dorsal horn of CNS (central nervous system) and stimulate primary afferent nociceptors, thus initiate pain response and writhing syndrome (Bley et al. 1998; Le Bars et al. 2001). In this study, MEPSS significantly ($p < 0.001$) reduced the number of abdominal constriction or writhing induced by acetic acid in the right hind paw of mice (Fig. 2). This result clearly indicates that the antinociception produced by the extract is due to its inhibition of endogenous

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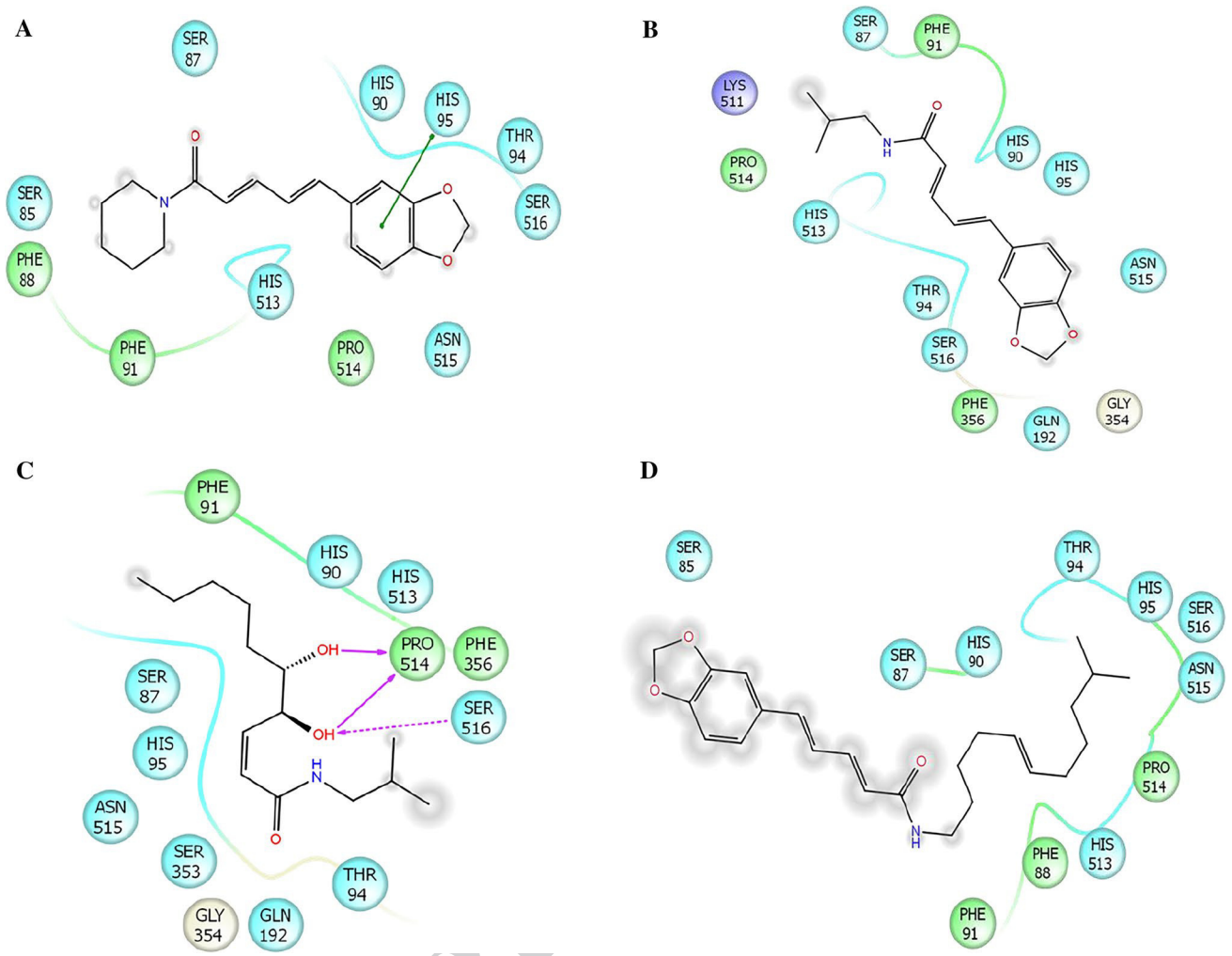

Fig. 3 2D interactions of the piperine (**a**), piperlonguminine (**b**), sylvamide (**c**), and sylvatine (**d**) with the active site of COX-1 (PDB ID: 2OYE). Colors indicate the residue (or species) type: Red-acidic (Asp, Glu), Green-hydrophobic (Ala, Val,Ile, Leu, Tyr, Phe, Trp, Met, Cys, Pro), Purple-basic (Hip, Lys, Arg), Blue-polar (Ser, Thr, Gln, Asn, His, Hie, Hid), Light gray-other (Gly, water) and Darker gray-metal atoms. Interactions with the protein are marked with lines between ligand atoms and protein residues: Solid pink: H-bonds to the protein backbone, Dotted pink: H-bonds to protein side chains, Green: pi–pi stacking interactions, Orange: pi-cation interactions. Ligand atoms exposed to solvent are marked with gray spheres. The protein "pocket" is displayed with a line around the ligand, colored with the color of the nearest protein residue. The gap in the line shows the opening of the pocket

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inflammatory mediators' synthesis or in the direct blockage of receptors.

Secondly, the formalin-induced paw licking test was carried out to determine whether the antinociceptive activity of MEPSS is of central or peripheral origin. The subcutaneous injection of formalin into the subplantar area of the right hind paw initiates biphasic nociceptive pain response. The early phase (0–5 min) is known as a neurogenic phase which begins with the direct stimulation of sensory afferent fibers (particularly C-fibers) and releases inflammatory mediators like bradykinin and substance P. Alternatively, the late phase (15–30 min) is known as an inflammatory phase that initiates by releasing several excitatory mediators like histamine, bradykinin, serotonin, prostaglandins in the peripheral tissues and interrupting the neuronal function of the spinal cord. Previous studies have been suggested that peripherally acting drugs such as aspirin, naproxen, diclofenac sodium, indomethacin, and dexamethasone inhibit the release of histamine, serotonin, bradykinin, prostaglandins and inhibit the late phase pain while centrally acting drugs (narcotics/opioids such as morphine, heroin, etc.) suppresses the nociception of both phases (early and late) in the formalin test (Wheeler-Aceto and Cowan 1991; Tjølsen et al. 1992; França et al. 2001; Yin et al. 2016). Results of this study show that MEPSS significantly and dose-dependently reduced the formalin induced nociceptive responses of both phases (Table 2). Accordingly, it can be said that the extract possesses both central and peripheral antinociceptive effects.

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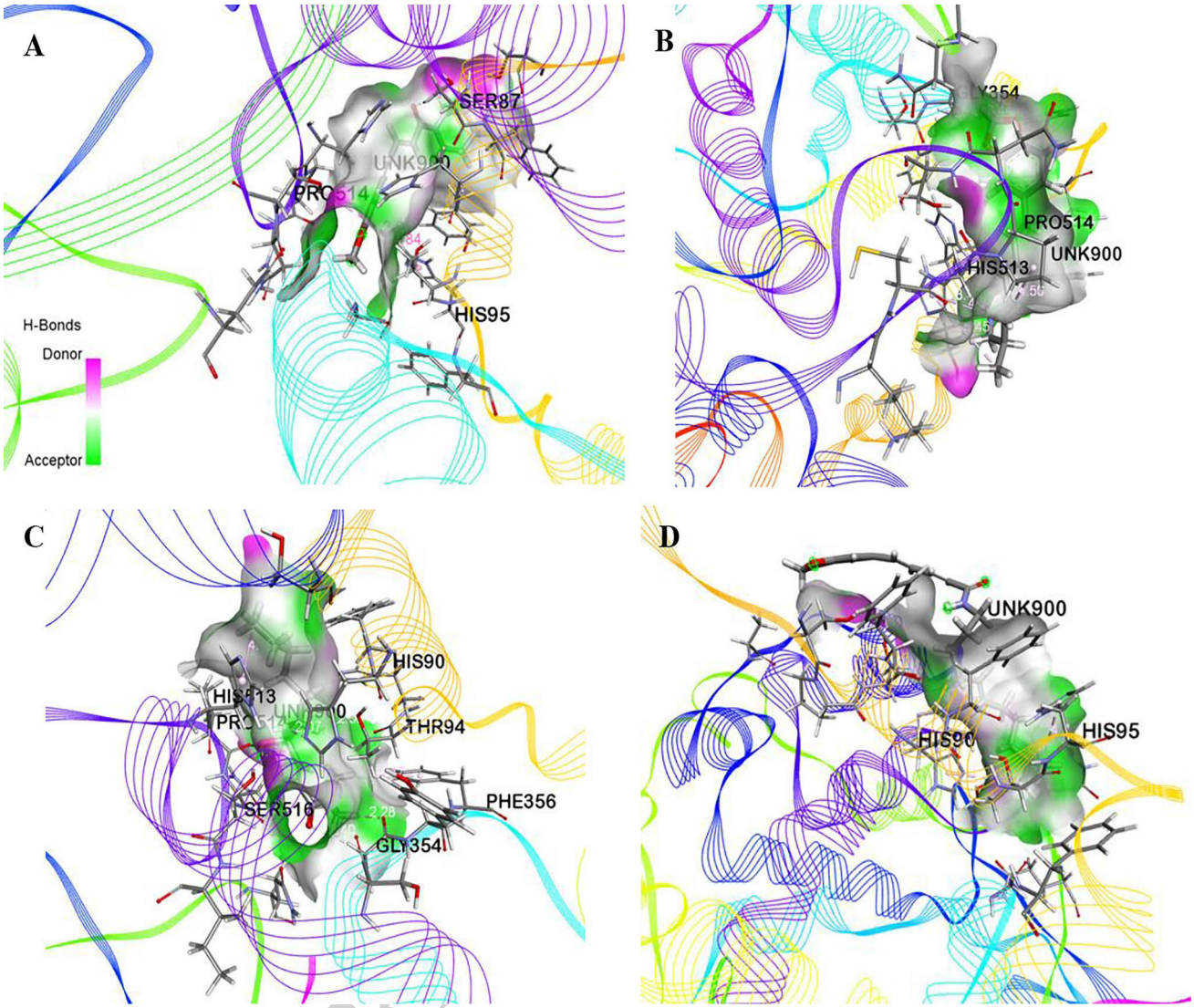

Fig. 4 Best ranked pose of (**a**) piperine, **b** piperlonguminine, **c** sylvamide, and **d** sylvatine in the binding pocket of COX-1 (PDB ID: 2OYE)

A previous qualitative phytochemical study of MEPSS exposed the presence of flavonoids, tannins, alkaloids, saponins and also our quantitative phytochemical study indicated the highest amount of phenol (93.39 mg) and flavonoids (53.74 mg) contents (Table 1) in the extract. It was reported previously by several scientific studies that many phytoconstituents like alkaloids (Barik 1992; Chao et al. 2009), flavonoids (Liang et al. 1999), tannins (Ramprasath et al. 2006), and saponins (Choi et al. 2005) are responsible for the antinociceptive and anti-inflammatory activity of the plants. Flavonoids have the ability to blockage the arachidonic acid metabolism by inhibiting the release of pro-inflammatory mediators such as prostaglandins, histamine, serotonin, cyclooxygenase, lipooxygenase and cytokines like IL-8, IL-1β, and TNF-α (Vezza et al. 2016). In addition, several scientific studies also reported that plant materials containing piperine (Bang et al. 2009; Bukhari et al. 2013; Tasleem et al. 2014) and piperlonguminine (Silva et al. 2008) have antinociceptive and anti-inflammatory potentials. So, it might be possible that the presence of such phytochemicals (mostly flavonoids, saponins, and alkaloids) and bioactive compounds (piperine and piperlonguminine) in MEPSS may be responsible for the antinociceptive and anti-inflammatory properties.

To verify the anti-inflammatory activity of MEPSS, we conducted the carrageenan-induced paw edema test in mice since this is the most standard method for evaluating the anti-inflammatory effects of both natural and synthetic compounds. The formation of edema in paw caused by carrageenan is a biphasic event (Singh et al. 2010). The first phase of inflammation occurs within an hour of carrageenan injection which is associated with the release and action of

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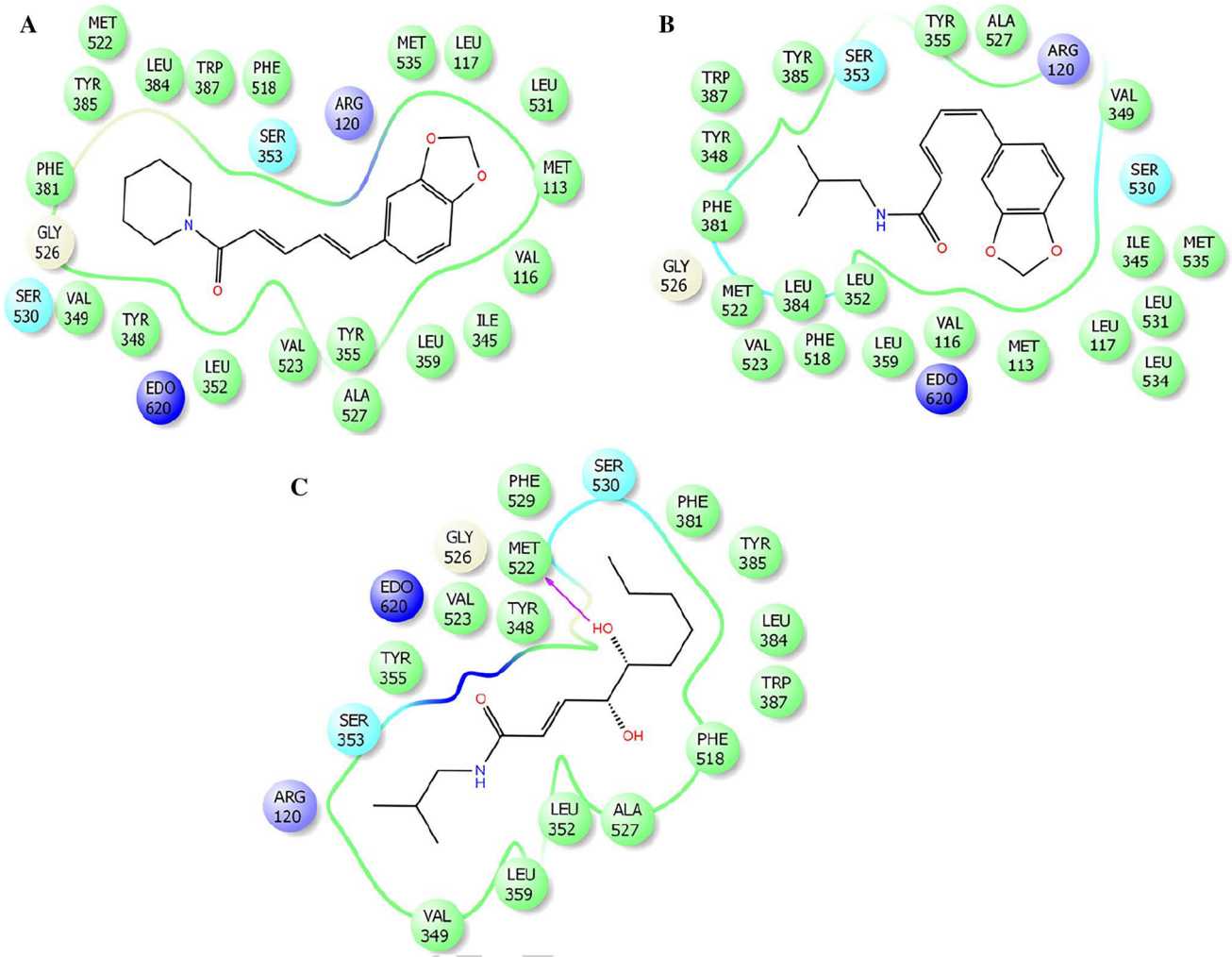

Fig. 5 2D interactions of the piperine (**a**), piperlonguminine (**b**), and sylvamide (**c**) with the active site of COX-2 (PDB ID: 3HS5)

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inflammatory mediators like serotonin and histamine while the late or second phase persists around two and half hours is primarily responsible for the prostaglandins synthesis and it's sensitive to the most clinically valid anti-inflammatory drugs (measured at the 3rd hour of the test) (Vinegar et al. 1969; Singh et al. 2010). From the result of anti-inflammatory activity in Table 3, it was found that there was no significant inhibition of paw edema in the first hours of study which clearly indicates not inhibition of serotonin and histamine. On the other hand, there is a significant percentage inhibition of paw edema, 32.30% and 49.23% at doses of 200 and 400 mg/kg, respectively, at the 3rd hour by the methanol extract. So, it can be concluded that the inhibitory effect of MEPSS on carrageenan-induced inflammation could be caused by inhibition of the COX enzymes leading to the blocking of prostaglandin synthesis.

At present, computational techniques like molecular docking have been commonly used for the theoretical prediction of ligand-target interactions to know the binding mode of active compounds against key enzymes/proteins and also to understand the possible molecular mechanism of the pharmacological activity of natural products/compounds (Adnan et al. 2019a, b). From this perspective, in silico molecular docking study was performed to understand better that mechanism of action and confirm their results with the experimental findings. In the present study, six major compounds of *P. sylvaticum* i.e. piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone were investigated against cyclooxygenase-1 and cyclooxygenase-2 enzymes and the docking scores obtained for all compounds have been reported in Table 4. Here, piperine, piperlonguminine, and sylvamide showed the best docking score against COX enzymes among all the compounds. Our docking study suggests that piperine, piperlonguminine, and sylvamide might be the responsible bioactive compounds for the potential antinociceptive and anti-inflammatory activities of the MEPSS. This finding is also full agreement with the previously reported data which stated that piperine (Bang

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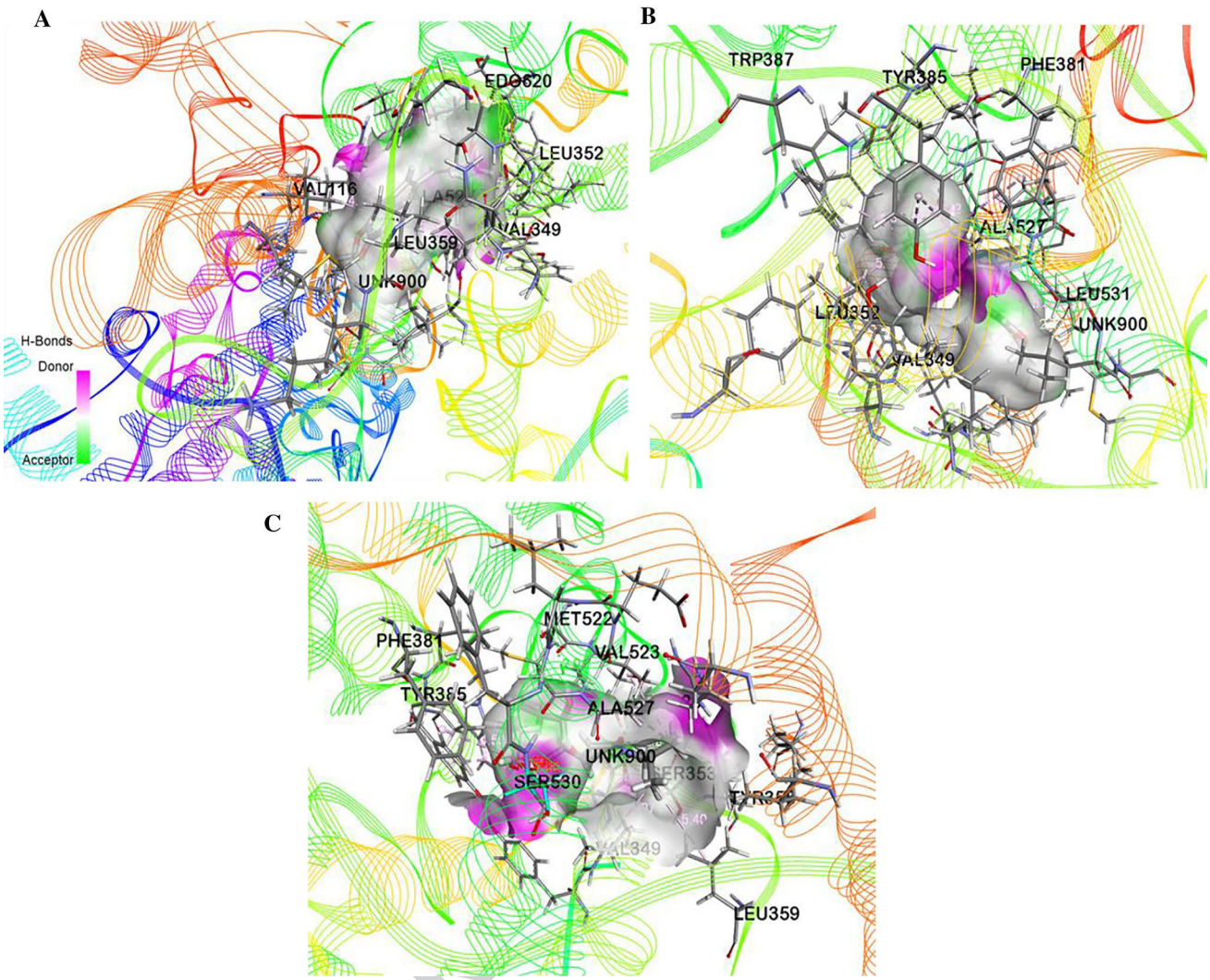

Fig. 6 Best ranked pose of (**a**) piperine, **b** piperlonguminine, and **c** sylvamide in the binding pocket of COX-2 (PDB ID: 3HS5)

Table 5 Physicochemical properties of the compounds for good oral bioavailability

Compounds	Lipinski rules					Lipinski's violations	Veber rules	
	MW	HBA	HBD	Log P	AMR		NRB	TPSA
Rule	< 500	< 5	≤ 10	≤ 5	40–130	≤ 1	≤ 10	≤ 140 Å^2
Piperine	285.34	3	0	3.04	85.47	0	3	38.78
Piperlonguminine	273.33	3	1	3.17	78.77	0	5	47.57
Sylvamide	257.37	3	3	2.16	74.26	0	9	69.55

MW molecular weight, *HBA* hydrogen bond acceptor, *HBD* hydrogen bond donor, *Log P* lipophilicity, *AMR* molar refractivity, *NRB* number of rotatable bond *TPSA* topological polar surface area

et al. 2009; Bukhari et al. 2013; Tasleem et al. 2014), piperlonguminine (Silva et al. 2008), and sylvamide (Kaur et al. 2019) have antinociceptive and anti-inflammatory effects.

Based on the highest docking score against cyclooxygenase enzymes (COX-1 and COX-2), we have selected three compounds i.e. piperine, piperlonguminine, and sylvamide to check their pharmacokinetic and toxicological properties as it is a major concern during the drug development process. Pharmacokinetic properties are considered to be crucial in the drug discovery process because it determines the essential features for successful oral drugs absorption to elimination. In addition, having poor pharmacokinetic

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Table 6 Toxicological properties of the compounds

Parameters	Compounds		
	Piperine	Piperlonguminine	Sylvamide
Ames toxicity	Non Ames toxic	Non Ames toxic	Non Ames toxic
Carcinogens	Non-carcinogenic	Non-carcinogenic	Non-carcinogenic
Acute oral toxicity	III	III	III
Rat acute toxicity	2.7129	2.5940	1.7848

Category-III means (500 mg/kg > LD_{50} < 5000 mg/kg)

properties means fail to commercialize a drug so it is more important to check its proprieties initially which totally depends on chemical descriptors of the molecules (Shahinozzaman et al. 2018). From this view, the SwissADME online server was used to calculate the pharmacokinetic properties (ADME: absorption, distribution, metabolism, elimination) of the selected compounds based on Lipinski's rule of five. According to Lipinski's rule of five, orally administered drugs or tested compounds should have a molecular weight of less than 500 amu, Lipophilicity value, Log$P \leq 5$, Hydrogen bond acceptor sites < 5, and Hydrogen bond donor sites ≤ 10. Any drugs or compounds which violate this rule possibly will have problems with bioavailability. Our present study exhibited that none of the phytocompounds violate these rules, which indicates good oral bioavailability (Table 5). In addition, a study conducted by Veber et al. suggested that a compound or drug should have the number of rotatable bonds (NRB) ≤ 10 and topological polar surface area (TPSA) value ≤ 140 $Å^2$ whereas NRB expresses the molecular flexibility of a molecule for suitable drugs and TPSA is involved in passive molecular transport of drugs through membranes (Shahinozzaman et al. 2018). Result of this study showed that all the compounds satisfied this rule which indicates that they are a suitable candidate for drugs and can transport through membranes. Toxicological properties of the selected compounds were also determined by the admetSAR online server. Our study showed that none of the compounds posed a risk of ames toxicity, carcinogenicity, acute oral toxicity, and rat acute toxicity (Table 6). Therefore, all three compounds are considered to be safe and orally bioavailable from a druggable point of view. Additionally, our acute toxicity study revealed that there is no mortality, abnormal behavioral and neurological changes at the doses up to 2000 mg/kg, suggesting that MEPSS has a low toxicity profile.

Conclusions

In conclusion, results of the present study demonstrated that MEPSS possess significant and dose-dependent antinociceptive and anti-inflammatory activities, which supports it uses in traditional medicine. These activities might be attributed to the occurrence of high phenolic contents and a number of phytochemicals such as flavonoids, alkaloids, saponins, and tannins that acts individually or collectively. Our molecular docking study showed that piperine, piperlonguminine, and sylvamide have a higher binding affinity towards COX enzymes for antinociceptive and anti-inflammatory activity. Moreover, ADME/T study revealed that these three bioactive phytocompounds are safe and orally bioavailable from a druggable point of view. Therefore, it can be concluded that these compounds could be a good source for the development of new antinociceptive and anti-inflammatory agents that warrants further study to reveal their in-depth molecular mechanism of action in animal models.

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Author's contributions Md. Nazim Uddin Chy, Md. Adnan and Arkajyoti Paul conceived and designed the experiments. Akash Kumar Rauniyar and Md. Moksadul Amin helped to write the original draft and contributed to data analysis. Md. Nazim Uddin Chy, Md. Adnan, Kaniz Farhana, Fayejun Nesa, Muazzem Ahmad Sany, Mohammad Akramul Hoque Tanim, and Tanvir Iqram Siddique carried out experimental works, analyzed and interpreted experimental results and wrote the manuscript. Md. Nazim Uddin Chy, Arkajyoti Paul, and Mohuya Majumder performed the computational study and wrote the relevant potion. This study was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical statements This study was carried out in accordance with the internationally accepted principle for proper use of laboratory animals namely National Institutes of Health and the International Council for Laboratory Animal Science. The present study protocol was reviewed and approved by the "P&D committee" of the Department of Pharmacy, International Islamic University Chittagong, Bangladesh with a reference number: Pharm-P&D-61/08'16-125.

Conflicts of interest Md. Nazim Uddin Chy has no conflict of interest. Md. Adnan has no conflict of interest. Akash Kumar Rauniyar has no conflict of interest. Md. Moksadul Amin has no conflict of interest. Mohuya Majumder has no conflict of interest. Md. Sahidul Islam has no conflict of interest. Shanta Afrin has no conflict of interest. Kaniz

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Farhana has no conflict of interest. Fayejun Nesa has no conflict of interest. Muazzem Ahmad Sany has no conflict of interest. Mohammad Akramul Hoque Tanim has no conflict of interest. Tanvir Iqram Siddique has no conflict of interest. Arkajyoti Paul has no conflict of interest.

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