

RESEARCH ARTICLE

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Antidiarrheal activity of methanol extract of *Piper sylvaticum* (roxb.) stem in mice and in silico molecular docking of its isolated compounds



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ABSTRACT

Background: *Piper sylvaticum* (Roxb.), is commonly used in traditional medicine to treat a number of disease like in asthma, diarrhea, chronic cough, cold, piles, tuberculosis, and wounds. In this study, we investigated the antidiarrheal activity of methanol extract of *P. sylvaticum* stem (Met.PSS) in animal models. Later, molecular docking study was performed to better understand its molecular mechanism and to determine the potent phytochemicals of this plant for the antidiarrheal property.

Methods: The stems were extracted with methanol and subjected to *in vivo* antidiarrheal study using the castor oil-induced diarrhea and castor oil induced enteropooling tests in animal models. And then, *in silico* molecular docking study was performed using Schrödinger suite Maestro v10.1.

Results: Met.PSS exhibited a dose-dependent and statistically significant antidiarrheal activity in both castor oil-induced diarrhea and enteropooling tests at the doses of 200 and 400 mg/kg. Additionally, our molecular docking analysis exhibited that four compounds viz. piperine, piperlonguminine, sylvamide, and sylvatine have the best binding affinity against the target enzyme (M3 muscarinic acetylcholine receptor) in comparison to reference drug Loperamide.

Conclusions: The present study suggests that Met.PSS possess significant antidiarrheal activity which could be related to the presence of various secondary plant metabolites or phytochemicals. Additionally, the phytochemicals, i.e., piperine, piperlonguminine, sylvamide, and sylvatine were found to be most effective in molecular docking study.

Keywords: *Piper sylvaticum*, Antidiarrheal, Castor oil, Enteropooling, Molecular docking

BACKGROUND

Plant-derived natural products play a vital role in the development of new therapeutic agents. They are the important sources of various bioactive compounds with diverse pharmacological properties. In recent years, the interest in the study of medicinal plants as a source of pharmacologically active compounds has increased worldwide. Furthermore, herbal medicines have a high demand in developing countries for primary health care due to a wide range of biological and medicinal properties with higher safety margins and lower costs. It has been reported that approximately 80% of the world population, especially from the developing countries, are directly relying on traditional medicines, mainly plant-based, to fulfill their primary health care needs.^{1,2} However, numerous plants have

not been studied yet for their claimed biological properties. On the other hand, it is estimated that around 500,000 species of medicinal plants exist on the earth and only a small percentage (1% to 10%) is used as food by humans and other animal species together. Besides, plant-derived natural products have been elaborated within living systems; they are often perceived as showing more 'drug-likeness' and biological friendliness than totally synthetic molecules, making them good candidates for further drug development.^{3,4}

Piper sylvaticum Roxb., (Family: Rubiaceae), is a climbing herb, commonly known as Pahari pipul (Hindi), Pahari peepal (Folk), and mountain long pepper (English). It is mainly distributed in tropical and sub-tropical regions. This plant has

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several therapeutic applications in the practice of traditional medicine in asthma, diarrhea, chronic cough, cold, piles, tuberculosis, wounds, headache, rheumatic pain, dyspepsia and indigestion.^{5,6} The previous phytochemical study has been reported the presence of various phytoconstituents like piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, β -sitosterol, and sylvone.⁷ There is some research for this plant in which elevated antioxidant activity⁸ is reported from roots and fruits, and the recent pharmacological study revealed that the plant possesses significant anthelmintic activity.⁹ Even though the plant, *P. sylvaticum* has several important medicinal properties, until now, no studies have been performed to investigate its antidiarrheal property. For that reason, this set of studies with the use of experimental (animal model) and computational approaches (*in silico* molecular docking) was performed to examine the antidiarrheal activity of methanol extract of *P. sylvaticum* stem (Met.PSS) for the first time.

METHODS

Drugs and chemicals

Methanol was obtained from Merck (Darmstadt, Germany) and Tween-80 from BDH Chemicals Ltd. Loperamide was purchased from Square Pharmaceuticals Ltd. (Dhaka, Bangladesh), and castor oil from WELL's Health Care (Madrid, Spain). All chemicals and drugs used in this investigation were of analytical grade

Plant material collection, identification, and preparation of methanol extract (Met.PSS)

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. 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 (Met.PSS). 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 $25\pm 2^\circ\text{C}$; 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.

Assessment of antidiarrheal activity of Met.PSS

For the assessment of antidiarrheal activity, two different antidiarrheal models namely castor oil induced diarrhea and castor oil-induced entero-pooling tests were used. The first one (castor oil induced diarrhea) is very helpful to assess the overall possible antidiarrheal effect of the plant material. And this was followed by the attempt of investigating the antidiarrheal mechanism of action of the plant extract i.e. either by inhibition of intestinal transit and/or antisecretory activity.

Castor oil induced diarrhea test in mice

The antidiarrheal activity of Met.PSS was carried out according to the protocol previously described by Awouters et al.¹⁰ with some modifications. Only animals which were found diarrheic when they have taken 0.5 ml castor oil in the initial screening test were included in this study. Briefly, mice fasted for 24 h and were randomly divided into four groups of six mice in each group (n = 6). Group-I was administered 10 ml/kg of Tween-80 (1% Tween-80 in distilled water) orally and served as a negative control (NC). Group-II was administered Loperamide (5 mg/kg, body weight, p.o.) and served as a reference standard drug (RSD), while Group-III and IV were administered an oral dose of Met.PSS at 200 and 400 mg/kg body weight respectively. One hour after administration of the test doses, the mice received 0.5 ml castor oil orally to induce diarrhea, and they were individually placed in cages, the floor of which was lined with blotting paper for observation of the number and consistency of fecal droppings. The total numbers of both dry and wet feces excreted were counted every 60 min for a period of 4 h, and the papers were changed every hour after each evaluation. The mean number of diarrheic feces pooled by the control group was considered as 100%. For all the groups the percentage inhibition of diarrhea (%) was calculated compared to the negative controls by using the following equation: inhibition (%) = [(TD control - TD test groups) / TD control] \times 100, where TD control = total number of diarrheal feces of the negative control group; TD test groups = total number of diarrheal feces of the test groups or reference standard drug.

Castor oil-induced enteropooling test in mice

This study was conducted by the method described by Robert et al.¹¹ In this study, the mice were fasted for 24 h but had free access to water and randomly divided into four groups of six mice each (n = 6). Group-I received normal saline 2 ml/kg body weight (p.o.) and served as a negative control (NC). Group-II received Loperamide (5 mg/kg, body weight, p.o.) and served as reference standard drug (RSD), while Group-III and IV were administered an oral dose of Met.PSS at 200 and 400 mg/kg body weight respectively. Briefly, one hour after administration of the test doses, 0.5 ml of castor oil was administered orally to each animal to induce diarrhea. Then 2 h later, the mice were sacrificed by an overdose of chloroform anesthesia, and the small intestine was ligated both at the pyloric sphincter and at the ileocecal junctions and dissected out. The small intestine was weighed (g) and the volume of intestinal contents (ml) was measured by milking into a graduated tube. The intestines were reweighed and the differences between full and empty intestines were calculated.

Selection of compounds for molecular docking study

Piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone were selected based on the availability as major compounds through literature review.⁷ The chemical structures of the compounds were downloaded from the PubChem database and docking was performed as we described previously.¹²

Ligand and protein preparations

The structures of six major representative compounds i.e., 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) were obtained from PubChem database whereas the ligands were prepared by the LigPrep tool embedded in Schrödinger suite Maestro v 10.1 (LLC New York, NY, USA), neutralized at pH 7.0 ± 2.0 by Epik 2.2, and minimized using force field OPLS_2005.

On the other hand, three dimensional X-ray crystal structures of the proteins used for this investigation were obtained from the Protein Data Bank (RCSB PDB): M3 muscarinic acetylcholine receptor (PDB: 4U14). The Protein Preparation Wizard of the Schrödinger suite Maestro v 10.1 was used to prepare and refine the crystal structures. Charges and bond orders were assigned, hydrogens added

to heavy atoms and selenomethionines and selenocysteines converted into methionines and cysteines respectively, followed by removing all water molecules. Using force field OPLS_2005, minimization was performed to set a maximum heavy atom RMSD to 0.30 Å.

Grid generation and molecular docking

The receptor grid generation and molecular docking experiments were performed using Glide (Schrödinger suite Maestro v 10.1).¹³ For each protein, a grid was produced using default parameters of van der Waals scaling factor 1.00 and charge cut-off value 0.25 subjected to the OPLS_2005 force field. Additionally, a cubic box of definite dimensions centered on the centroid of the active site residues was generated for receptor and the size of the box was set to 14 Å × 14 Å × 14 Å for docking. Here, the docking experiments have been carried out with the Standard Precision (SP) scoring function of Glide and only the best scoring pose with docking score was note down for each ligand.

Statistical analysis

Results were presented as mean ± SEM. SPSS software (v 20) was used for statistical data analysis, and all comparisons were made by using one-way ANOVA followed by Dunnett's test whereas p-value of less than 0.05 was considered as statistically significant.

RESULTS

Castor oil-induced diarrhea

Results for castor oil induced diarrhea are shown in [Table 1](#). In the castor oil-induced diarrheal model, Met.PSS showed significant inhibition of diarrhea at both doses (200 and 400 mg/kg), in a dose-dependent fashion. The maximum inhibitory effect was found at a dose of 400 mg/kg (40.62%), which is comparable with the standard drug Loperamide (65.62%). Additionally, Met.PSS produced a noticeable reduction in defecation numbers at doses of 200 mg/kg (49.31%) and 400 mg/kg (56.16%) respectively, compared to the control group. Moreover, the reduction of diarrheal feces was also showed dose-dependently, with the best antidiarrheal effect observed at the higher dose of 400 mg/kg, compared to the reference standard drug (Loperamide).

Castor oil induced enteropooling

In the castor oil induced gastrointestinal enteropooling test, the Met.PSS reduced the volume and weight of the intestinal contents significantly in a dose-dependent manner. The results revealed that

Table 1 The effect of *P. sylvaticum* extract on feces count in castor oil-induced diarrhea in mice

Treatment (mg/kg)	Total Number of Feces	% Inhibition of Defecation	Total Number of Diarrheal Feces	% Inhibition of Diarrhea
NC	14.60 ± 0.74		6.40 ± 0.81	
RSD (5)	5.40 ± 0.24***	63.01	2.20 ± 0.20***	65.62
Met.PSS (200)	7.40 ± 0.67***	49.31	4.80 ± 0.48	25.00
Met.PSS (400)	6.40 ± 0.50***	56.16	3.80 ± 0.58*	40.62

Significantly different when compared with that of the control group at * $p < 0.05$, *** $p < 0.001$. Values are expressed as mean ± SEM ($n = 6$). NC, Negative control; RSD, Reference standard drug; Met.PSS, Methanol extract of *P. sylvaticum* stem.

Table 2 The effect of *P. sylvaticum* extract on castor oil-induced enteropooling in mice

Treatment (mg/kg)	Volume of Intestinal Content (mL)	% Inhibition	Weight of Intestinal Content (gm)	% Inhibition
NC	0.71 ± 0.02		0.51±0.02	
RSD (5)	0.29 ± 0.01***	58.87	0.26±0.01**	49.41
Met.PSS (200)	0.46 ± 0.05*	34.64	0.39±0.03	22.56
Met.PSS (400)	0.38 ± 0.03***	46.47	0.34±0.02**	33.85

Significantly different when compared with that of the control group at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values are expressed as mean ± SEM ($n = 6$). NC, Negative control; RSD, Reference standard drug; Met.PSS, Methanol extract of *P. sylvaticum* stem.

Table 3 Docking score of piperine, piperlonguminine, sylvamide, sylvatine, sylvatesmin, and sylvone against M3 muscarinic acetylcholine receptor (PDB ID: 4U14).

Compound name	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)
Piperine	-7.62	-42.26	-22.75
Piperlonguminine	-5.63	-24.80	-11.57
Sylvamide	-2.03	-17.15	-13.56
Sylvatesmin	-	-	-
Sylvatine	-5.42	-29.51	-20.5
Sylvone	-	-	-
Loperamide	-7.32	-41.63	-42.65

the percentage inhibition of intestinal content was 34.64% and 46.47% at doses of 200 and 400 mg/kg, respectively whereas reference drug Loperamide showed 58.87% inhibition as shown in Table 2. The highest effect on both volume and weight of intestinal contents was observed at 400 mg/kg of the Met.PSS.

Molecular docking study for antidiarrheal activity

Results of docking study for antidiarrheal activity are presented in Table 3, and the docking figures are shown in Figures 1-2. In the present study, M3 muscarinic acetylcholine receptor (PDB: 4U14) involved in intestinal motility were used to search the possible mechanism of the antidiarrheal effect of Met.PSS. From the result, it is clear that piperine showed the highest docking score against the muscarinic acetylcholine receptor (-7.62 kcal/mol), followed by piperlonguminine (-5.63 kcal/

mol), sylvatine (-5.42 kcal/mol), and sylvamide (-2.03 kcal/mol). Among all compounds, three, namely piperine, piperlonguminine, and sylvatine exhibited better docking scores in comparison to the reference drug Loperamide (-7.32 kcal/mol). Additionally, piperine exhibited better docking score than the reference drug Loperamide (-7.32 kcal/mol). However, two compounds, namely sylvatesmin and sylvone did not dock with the muscarinic acetylcholine receptor.

Study of the docking fits of each compound suggested various interactions between the ligands and the target proteins or enzymes. Piperine interacts with the M3 muscarinic receptor (PDB: 4U14) through one hydrogen bond to Trp503 (docking score -7.62 Kcal/mol). Piperlonguminine interacts with the same enzymatic pocket through the formation of one hydrogen bond with Ser151 and by forming three pi-pi stacking interactions with

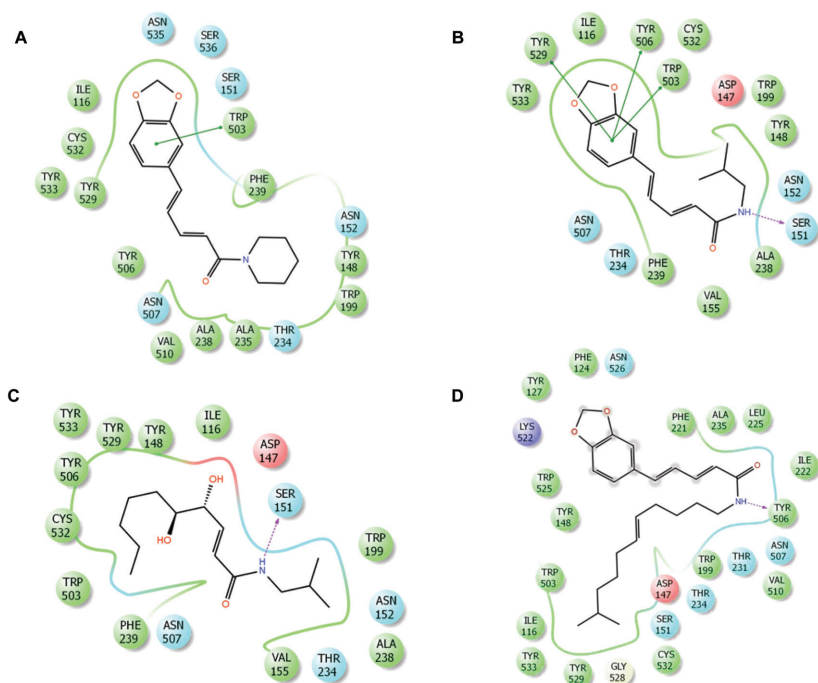


Figure 1 2D interactions of the best pose found for (A) Piperine, (B) Piperlonguminine, (C) Sylvamide, and (D) Sylvatine docked to the M3 muscarinic acetylcholine receptor (PDB ID: 4U14). Colors indicate the residue (or species) type: Red-acidic (Asp, Glu), Green-hydrophobic (Ala, Val, Ile, Leu, Tyr, Phe, Trp, Met, Cys, Pro), Purple-basic (His, 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.

Tyr529, Tyr506, and Trp503 residues (docking score -5.63 kcal/mol) while sylvamide interacted with the same enzymatic pocket by establishing one hydrogen bond with Ser151 (docking score -2.03 kcal/mol). In addition, sylvatine interacted by forming one hydrogen bond with Tyr506 (docking score -5.42 kcal/mol). However, sylvatesmin and sylvone did not show any interactions with M3 muscarinic receptor whereas the reference drug loperamide interacts with M3 muscarinic receptor through two π - π stacking interactions with Trp525, with a docking score of -7.32 kcal/mol.

DISCUSSION

Medicinal plants contain a huge variety of bioactive compounds for natural protection against microbial, insect or pest attack, and also chosen to treat

GIT (gastrointestinal) disorders, like, constipation and diarrhea. And most of these compounds have been used in the forms of plant extracts or whole plants for medical applications in human since plants are the natural reservoir of many therapeutic activities like antidiarrheal, anthelmintic, antifungal, antimicrobial, sedative, analgesics anti-inflammatory agents, etc.¹⁴ Additionally, these bioactive compounds have different mechanisms than synthetic drugs and could be of clinical importance to improve patient health care. Therefore, the acceptance of medicine from such plant origin as an alternative approach for health care and this approach is increasing day by day. With this view, the present study was carried out to investigate the antidiarrheal activity of the methanol extract of *P. sylvaticum* stem (Met.PSS) in animal models followed by *in silico* molecular docking analysis.

Diarrhea is defined as a disorder that is described by the discharge of semisolid or watery fecal matter from the bowel three or more times in a day. It is one of the major health problems globally, particularly for children under 5 years of age. Moreover, it is one of the leading causes of morbidity and mortality in the people of developing countries.¹⁵ Though some effective drugs are available throughout the world, searching of new antidiarrheal agents from plant origin are still encouraged by the World Health Organization (WHO) due to its safety, cost-effective and easy availability. In antidiarrheal activity screening, castor oil is known as diarrhea inducer. Its active metabolite, ricinoleic acid, which stimulates peristaltic activity in the upper part of the small intestine, leading to changes in electrolyte permeability of the intestinal mucosa.¹⁶ Ricinoleic acid also causes irritation and inflammation of the intestinal mucosa leading to the release of several inflammatory mediator substances including prostaglandins, nitric oxide, histamine, and tachykinins, etc. that eventually increase gastrointestinal motility, net secretion of water & electrolytes and induce diarrhea.^{11,17}

For the evaluation of *in vivo* antidiarrheal activity of Met.PSS, we began our investigation with castor oil induced diarrheal model which is a broadly accepted method to evaluate the antidiarrheal property of the plant extract. In the castor oil induced diarrheal test, the Met.PSS showed a significant reduction in the rate of defecation and consistency of feces in mice which was comparable with that obtained by the standard drug Loperamide. It decreased the frequency of discharge of feces, reduced the number of wet stools and inhibited the severity of diarrhea. Secondly, we performed a castor oil-induced entero-pooling test to investigate the antidiarrheal activity of Met.PSS. In this study, the extract significantly

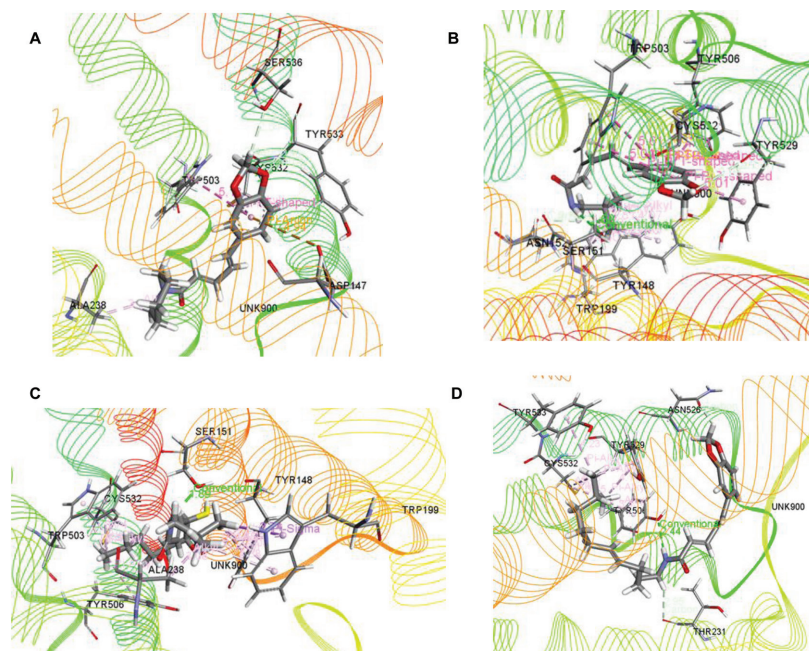


Figure 2 Best ranked pose of (A) Piperine, (B) Piperlonguminine, (C) Sylvamide, and (D) Sylvatine in the binding pocket of M3 muscarinic acetylcholine receptor (PDB ID: 4U14).

and dose-dependently reduced both the weight and volume of intestinal contents induced by castor oil proving its anti-enteropooling activity. Besides, the effects produced by the extract were also found to be nearly similar to those of reference standard drug, Loperamide group.

A previous phytochemical study of the Met.PSS revealed the presence of several plant secondary metabolites including alkaloids, carbohydrates, flavonoids, tannins, and saponins. The antidiarrheal properties of Met.PSS may be due to the individual or combined effects of tannins, alkaloids, saponins, and flavonoids.¹⁸ The phytochemicals namely flavonoids and alkaloids are also known to inhibit the release of autacoids and prostaglandins, thereby inhibiting secretion induced by castor oil.^{19,20} Tannins^{21,22} have been reported to reduce the irritability of the bowel, resulting in a reduction of peristaltic movements and intestinal secretions while saponins²³ also exhibit antidiarrheal activity by inhibiting the release of histamine. Therefore, it could be suggested that the presence of these phytochemicals in the Met.PSS is responsible for the observed pharmacological effect.

Computational studies have been effectively used for the prediction of ligand-target binding affinity and to better understand the possible molecular mechanism of the pharmacological responses. Keeping this in view, molecular docking study was performed to understand comprehensively that mechanisms and to confirm their findings with the experimental results. In the present

study, six major compounds of *P. sylvaticum* were investigated against M3 muscarinic acetylcholine receptor (PDB ID: 4U14) and the docking scores obtained for all compounds have been reported in Table 3. Our molecular docking study suggests that piperine, piperlonguminine, sylvamide, sylvatine may be the responsible bioactive phytochemicals for the potential antidiarrheal activity of the plant, although further *in vivo* studies are necessary to explore their in-depth mechanism of action. This finding is also consistent with the previously reported data since an earlier study stated that piperine²⁴ and piperlonguminine²⁵ are responsible for antidiarrheal activities.

CONCLUSION

In short, our present study demonstrated that Met. PSS possesses significant antidiarrheal activity. This activity might be attributed to the presence of bioactive phytochemicals (piperine, piperlonguminine, sylvamide, sylvatine) and also abundant phytochemicals such as alkaloids, flavonoids, saponins, and tannins that act individually or collectively. Our molecular docking study also showed that piperine, piperlonguminine, sylvamide, and sylvatine have a higher binding affinity with M3 muscarinic acetylcholine receptor among all compounds for antidiarrheal activity. So, it can also be concluded that piperine, piperlonguminine, sylvamide, and sylvatine could be a noble source for the development of new antidiarrheal agents that deserves further research to explain their particular molecular mechanism of actions.

LIST OF ABBREVIATIONS

Met.PSS: Methanol extract of *p. sylvaticum* stem; p.o.: per oral; RMSD: root-mean-square deviation; ANOVA: Analysis of variance; SEM: standard error of mean; SPSS: statistical package for social science

DECLARATIONS

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Availability of data and materials

All data and materials are presented in the manuscript.

Consent for publication

Not applicable.

Author's contributions

MTH, NC, MRS, and FT conceived and designed the experiments. NC, MTH, MRS, FT, MM, MMAH, MZH, AAM, MMR, MRUP carried out all the experimental works, collected, analyzed and interpreted the experimental data, and drafted the original manuscript. NC, MTH, ME, MAK performed the computational study and wrote the final manuscript. This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest

Ethics approval and consent to participate

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 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.

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