In-vitro, in-vivo and molecular docking analysis of *Alternanthera philoxeroides* root phytocompounds targeting COX enzymes for analgesic, anti-inflammatory and antipyretic activity

Faruk Alam[1], Moidul Islam Judder[2], Mohidul Islam[3]*, Moksood Ahmed Laskar[3], Alindam Ghosh[4], Avik Dutta[4] and Soma Das[5]

[1]*Faculty of Pharmaceutical Science, Assam down town University, Panikhaiti, Guwahati-781026, Assam.*
[2]*M.R. College of Pharmaceutical Sciences and Research, Bira, Balisha, West Bengal, 743234*
[3]*Bengal College of Pharmaceutical Technology, Ward No. 8, Cinema Hall, by pass road, Notunpally, Dubrajpur, West Bengal 731123*
[4]*DMBH Institute of Medical Sciences, Dadpur, West Bengal 712305*
[5]*Himalaya Pharmacy Institute, Majitar, East-Sikkim 737136, India.*
[*]*Email: mohidulislam11302@gmail.com*

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ABSTRACT

Alternanthera philoxeroides (Mart.) Griseb. is traditionally used by tribal communities of Assam, India, for the treatment of headache, gastrointestinal disorders, dysentery, and asthma. The current study assessed the analgesic, anti-inflammatory, and antipyretic potential of the methanolic root extract of A. philoxeroides (MEAP) through in-silico, in-vitro, and in-vivo approaches, with emphasis on cyclooxygenase (COX) inhibition. Phytoconstituents investigation and spectroscopic analysis verified the existence of flavonoids and phenolic compounds. GC–MS profiling identified 23 constituents, notably 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one as well as 2-amino-5-[(2-carboxy) vinyl]-imidazole, which demonstrated strong affinity for binding toward COX-1 and COX-2 in-silico. MEAP at doses of 200 mg/kg and 400 mg/kg body weight exhibited significant dose-dependent analgesic activity, as evidenced by increased latency in the Eddy's hot plate test (59.22 ± 1.22%) and inhibition of writhing (65.06 ± 1.74%) induced by acetic acid. Anti-inflammatory potential was observed (58.93 ± 2.63%) induced by carrageenan. A pronounced antipyretic property was observed (82.58 ± 4.32%) in the method of pyrexia induced by Brewer's yeast. These findings indicate that MEAP exerts non-selective COX inhibitory activity, validating its traditional use and highlighting its potentiality as a natural remedy for inflammatory and pain-related conditions.

Keywords: *Alternanthera philoxeroides*, analgesic, anti-inflammatory, antipyretic, molecular docking

INTRODUCTION

Alligator weed (*Alternanthera philoxeroides* (Mart.) Griseb.), a group of family Amaranthaceae, is a non-woody summer ceaseless aquatic plant that reproduces vegetatively from the axillary buds at each node rather than setting viable seed when conditions are right. *A. philoxeroides* has an inordinate reputation in ethnobotanical aspects among various tribes in the Assam as well as in India. In many areas of Assam, the plant is consumed as a vegetable (mostly the young stem and leaf), and is used to treat dysentery and asthma, in

addition to being used to alleviate headaches. Before going to bed on a daily basis for a week, members of the Goreswar, Bodo, and Santhal tribes in the north-eastern region of India administer a mixture of young shoot and leaf juice with an equal volume of water for stomach pain (Dutta, 2015). The curative action of pain is achieved by inhibiting the synthesis of prostaglandins in the arachodenic acid pathway. In this pathway, Cyclooxygenase-1 (COX-1) enzyme and Cyclooxygenase-2 (COX-2) enzyme are responsible for the synthesis of prostaglandins (Alghamdi *et al*. 2023). COX-1 is intrinsically exhibited in majority of tissues, including stomach, kidneys, and platelets. It produces prostaglandins like PGE_2, PGI2, and TXA_2 to control essential physiological activities. In contrast, COX-2 is often absent but activated by inflammatory stimuli such as cytokines (IL-1, TNF-α) and lipopolysaccharides. It is in charge of producing prostaglandins, such as PGE_2 and PGI_2, which control fever, pain, and inflammation (Paulina *et al*., 2024). Non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin and ibuprofen inhibit COX-1, lowering protective prostaglandins and causing stomach ulcers and renal dysfunction (Panchal and Sabina 2023). Although COX-2 inhibitors like celecoxib relieve inflammation and discomfort with less gastrointestinal side effects, they may raise cardiovascular risk owing to lower PGI_2 , which has antithrombotic properties (Paulina *et al*., 2024). Hence, additional medication is required to overcome the side effects and so treatment become costly. Traditional and herbal remedies have become more popular for treating diseases because of the expensive cost and negative effects of allopathic medications (Ahmad and Sharma 2020). Thus, due to ethnomedicinal claims of *Alternanthera philoxeroides* (Mart.) Griseb. in several areas of Assam, the purpose of the research is to investigate COX-1 and COX-2 inhibitory property *in-vitro* as well as *in-vivo* assessment of analgesic, anti-inflammatory, and antipyretic effect.

MATERIALS AND METHODS

The experimental procedures were permitted by Institutional Animal Ethics Committee (IAEC) of Assam Down Town University, Guwahati, Assam, India (AdtU/IAEC/2022/10). The plant was authenticated as *Alternanthera phyloxeroides* (Mart.) Griseb. with the Accession no. GUBH19918. Roots the plant was collected by hand-pinking method from the village Fulgasa of the district Dhubri, Assam, India in the month of September 2020. The roots were air dried at room temperature and size reduced to form coarse as well as fine powder. Physico-chemical constants such as extractive values, moisture content and ash contents were determined (Sharma and Pracheta 2013; Sanket and Jitendra 2025) in Pharmacognosy laboratory, Assam down town University in the year 2021. Further, Powdered roots were extracted with methanol in the Soxhlet device (Kokate *et al.*, 2013). The methanol extract of *Alternanthera philoxeroides* (MEAP) was analyzed by phytochemical screening to detect phytoconstituents (Dalila *et al.* 2022; Islam and Alam, 2022).

Measurement of total flavonoid content (TFC)

Stock solution of standard drug rutin (1 mg/ml) was prepared. It was further diluted to prepare (10 µg/ml to 100 µg/ml) solutions. To 1 ml of each rutin solutions and 1 ml of MEAP (1 mg/ml), distilled water (4 ml) and 5% sodium nitrite (0.3 ml) were added. The reaction mixtures were incubated in the dark for 5 minutes with 10% aluminium chloride (0.3 ml). Then 1 ml 1M sodium hydroxide and 0.5 ml distilled water further added. After shaking the reaction mixture, UV-visible spectrophotometers assessed the solution's absorbance at 510 nm. TFC of MEAP was quantified by standard calibration curve (Sinaga *et al*., 2021).

Measurement of total phenolic content (TPC)

Mixed 0.5 ml of MEAP solution (1 mg/ml) and gallic acid solutions (10 µg/ml to 100 µg/ml) with Folin-Ciocalteu reagent (2 ml) and 7.5% sodium carbonate (4 ml). The reaction

combination was mixed and incubated for 30 minutes at normal temperature. The solutions' absorbance was recorded at 765 nm using a UV-visible spectrophotometer. MEAP phenolic content was estimated using the standard curve (Sinaga *et al*., 2021).

In-vitro anti-inflammatory effect of MEAP by egg albumin denaturation assay

Added 0.2 ml of albumin from eggs as well as 2.8 ml pH 6.5 phosphate buffer to a 2 ml aqueous solution of diclofenac sodium and MEAP (0.1 mg/ml to 0.4 mg/ml). The reaction mixture was heated to 70ºC for five minutes after being incubated at 37ºC for twenty minutes, and chilled. The control was double-distilled water. UV-Visible spectrophotometers recorded absorbance at 230 nm (Abbas *et al*., 2021). Finally, %inhibition was computed.

In-vitro anti-inflammatory effect of MEAP by Bovine serum albumin denaturation assay

1 ml aqueous solution of diclofenac sodium and MEAP (0.1 mg/ml to 0.4 mg/ml) was supplemented with bovine serum albumin (0.90 ml). For twenty-five minutes, the reaction mixture was then incubated at 25ºC. Further, added phosphate buffer (5 ml of pH 6.3) to each reaction combination and incubated in a water bath at 70ºC for fifteen minutes. UV-Visible spectrophotometer measured absorbance at 660 nm (Abbas *et al*., 2021). Finally, %inhibition was computed.

Phytochemical characterization of MEAP by GC-MS

MEAP phytoconstituents were analyzed by GC-MS on a 30-m × 0.25 mm × 0.25 µm RTX-5 capillary column. To optimize MEAP analysis using column alignment, the following parameters were used: carrier gas with flow rate of 1.5 ml/min, 260ºC injection temperature, 1 µl injection volume, split mode (1:100 operation mode), 40ºC retention for 2 minutes, 250ºC retention for 5 minutes, and 10ºC/min program temperature. The mass spectrometry detection approach used electron ionization with ion source and interface temperatures set at 200ºC and 250ºC, respectively. Mass-to-charge ratio (m/z) of ions was scanned from 40-700 at 0.5 second intervals and 1.5 scans/s (Huang *et al*., 2017).

In-silico molecular docking study for cyclooxygenase inhibitory property

3D arrangements of COX-1 enzyme and COX-2 enzyme were acquired from RCBS Protein Data Bank in PDB format. Removing heteroatoms optimized proteins and reduced energy. All water molecules, protein-bound medicines, and chains other than chain-A were removed and preserved in PDB. The chemical structure of MEAP compounds was sketched in ChemSketch (ACD laboratories) and saved as pdb files. Finally, Autodock Tools 1.5.6 docked chemicals to proteins. The program made ligands and proteins visible, removed water molecules, added Kolmann charges, assigned the atom to AD4 type, and docked against the appropriate proteins. Docking was visualized using Discovery Studio 2020 Client Visualizer (Shi *et al*., 2018).

Animal acclimatization and oral acute toxicity study of MEAP

Wistar albino rats were procured from Kolkata Saha Enterprises and housed at the animal facility of Assam down town University. The study was conducted in 2023 with prior approval from the IAEC of Assam down town University. Experimental animals in good health, both male and female of 140g to 160g were chosen. The animals were kept in clean, sterile polypropylene cages at 25±2°C, with a 12 hour cycle of light as well as dark and free access for normal food and water. Before the experimentations, the rats were separated for a week for environmental and handling acclimatisation after being randomly assigned to groups. Acute toxicity assessment was performed as per Organisation for Economic Co-operation and Development (OECD) 423 guidelines before experimental research (OECD, 2001). Three female rats were administered with MEAP at 2000 mg/kg of body weight in the volume 0.1ml/10gm of bodyweight by oral route using oral feeding cannula. Then, observed independently for the

initial 30 minutes, further, for 4 hours at 1 hour interval and periodically for 24 hours and then for 14 days regularly.

Analgesic activity of MEAP by Eddy's hot plate test

The analgesic efficacy of MEAP was assessed using Eddy's hot plate test with diclofenac sodium as described by Wahid *et al*., 2020. The rats' latency time was collected in seconds every 30 minutes for 2 hours to calculate %inhibition.

Analgesic activity of MEAP by acetic Acid induced writhing test

Analgesic impact was assessed in four groups of six male and female rats in the same ratio as described by Meegada *et al*., 2021. Oral diclofenac sodium (10 mg/kg body weight) and MEAP (200 mg/kg and 400 mg/kg body weight) were given 30 minutes before 0.75% v/v acetic acid induction at 1 ml/100g body weight. The dose of acetic acid was selected based on established protocols, as it produces a consistent and reproducible writhing response without significant mortality, enabling reliable assessment of peripheral analgesic activity (Meegada *et al*., 2021). Each group counted writhes for 15 minutes from 5 minutes after the acetic acid injection until 20 minutes and computed %inhibition.

Anti-inflammatory activity of MEAP by Carrageenan induced paw edema test

MEAP (200 mg/kg and 400 mg/kg body weight) and Indomethacine (10 mg/kg body weight) were administered orally to experimental rats (n=6). One hour following 1% carrageenan subcutaneous injection inferior in right hind paw plantar tissues caused paw edema. The inhibition percentage was calculated by measuring paw volumes with a Plethysmometer before 1% carrageenan administration and then for 4 hours at 1 hour intervals (Fitri *et al*., 2021).

Antipyretic activity of MEAP by Brewer's yeast induced pyrexia test

As per the method described by Alyas *et al*. (2022), subcutaneous injection of 15% Brewer's yeast caused pyrexia. Rats in groups (n=6) received oral MEAP (200 mg/kg and 400 mg/kg body weight) and paracetamol (10 mg/kg body weight). All groups' rats starting temperatures were taken before Brewer's yeast was given. 18 hours after yeast treatment, rectal temperatures were taken again. Experimental rats received paracetamol and MEAP at the appropriate dosages. The rectal temperatures of each group's rats were monitored using thermal probe thermometer for 4 hours at 1-hour intervals to compute %inhibition.

Statistical analysis

Data obtained were statistically analysezed by determining mean value and standard deviation. Also the data were processed for one way analysis of variance (ANOVA) followed by the Dunnett's post-hoc for conclusion of significance level. The significance of the data were considered $P < 0.05$ as significant, while $P < 0.01$ as moderately significant and $P < 0.001$ as highly significant.

RESULTS AND DISCUSSION

Physicochemical evaluation is a fundamental step in establishing the quality and authenticity of crude plant materials. In the present study, the moisture content (9.77 ± 0.41%) was observed to be within a permissible range, indicating adequate drying of the sample. Controlled moisture levels are essential to prevent microbial contamination and degradation, thereby ensuring better stability during storage. The extractive values provide an estimate of the nature and quantity of phytoconstituents present in the plant material. The water extractive value (0.94 ± 0.08%) suggests the presence of a limited amount of water-soluble constituents, whereas the alcohol extractive value (1.08 ± 0.08%) was comparatively higher, indicating a greater proportion of alcohol-soluble compounds. This difference reflects the predominance of polar to

non-polar constituents in the sample and highlights the suitability of alcohol as a solvent for extraction. Ash values serve as indicators of the inorganic content and possible contamination of the crude drug. The total ash value (10.98 ± 0.56%) represents the overall mineral content, while the acid-insoluble ash (8.16 ± 0.25%) points towards the presence of siliceous matter such as soil or sand. Additionally, the water-soluble ash (5.25 ± 0.28%) denotes the fraction of inorganic components that are soluble in water, which may include essential mineral elements. The consistency of the observed findings supports the purity of the sample and its suitability for further pharmacological investigations.

Phytochemical analysis showed that MEAP contained numerous phytoconstituents includes glycoside, carbohydrate, fixed oils and lipids, terpenoids, flavonoids, tannins, and phenolic chemicals, while alkaloids and proteins were absent (Table 1). The abundance of phenolics and flavonoids suggests potential antioxidant and anti-inflammatory properties, whereas tannins and terpenoids and glycosides may further contribute to diverse biological activity (Hussain *et al.,* 2016; Sharifi-Rad *et al.,* 2017). Hence, these findings highlight the presence of diverse bioactive compounds that could be responsible for the therapeutic potential of MEAP.

MEAP showed the presence of significant amount of flavonoids and phenolic compounds. The total flavonoid content (83.78±1.51 mg/g of extract, expressed as Rutin equivalent) and total phenolic content (70.81±0.72 mg/g of extract, expressed as Gallic acid equivalent) suggest that MEAP could serve as a promising source of natural antioxidant and anti-inflammatory compounds. Further, MEAP was confirmed for anti-inflammatory efficacy *in-vitro* by egg albumin and bovine serum albumin denaturation assays (Figure 1). The MEAP demonstrated dose-dependent and predictable anti-inflammatory efficacy. As the concentration increased from 100 to 400 µg/ml, a gradual rise in inhibitory activity was observed. In comparison to diclofenac sodium, MEAP demonstrated lower inhibition at all tested concentrations, although both exhibited a similar trend of increasing activity. This parallel response suggests a comparable mode of action.

MEAP showed 23 compounds on GC-MS chromatogram (Figure 2) such as (1) 1-Methoxy-2-propanol, (2) Methyltartronic acid, (3) 2-Methyl-1-propanol, (4) 2-(1,1-dimethylethyl)-3-ethyl-cis-oxirane, (5) Ethyl butanoate, (6) Pentyl acetate, (7) Ethyl hexanoate, (8) Cyclopropyl carbinol, (9) O-decyl-hydroxylamine, (10) 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, (11) 5-Methoxypyrrolidin-2-one, (12) Isoglutamine, (13) 2-Amino-5-[(2-carboxy)vinyl]-imidazole, (14) Tetradecamethyl-cycloheptasiloxane, (15) Methyl-6-oxoheptanoate, (16) 2-Acetamido-2-deoxy-d-mannolactone, (17) Propylphosphonic acid, fluoroanhydride, decyl ester, (18) Hexadecamethyl-cyclooctasiloxane, (19) 2,6,10,14-Tetramethyl-heptadecane, (20) Tetradecanoic acid, (21) 6,10,14-Trimethyl-2-Pentadecanone, (22) l-(+)-Ascorbic acid 2,6-dihexadecanoate and (23) Phytol. MEAP found to contained with active anti-inflammatory potentiality phyto-compounds like the "2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one" and "2-Amino-5-[(2-carboxy)vinyl]-imidazole" (Saxena and Rao 2021). These compounds while docked against COX-1 (PDB ID: 1EQG) and COX-2 (PDB ID: 3LN1) and showed profound binding affinity interacting with several amino acids of both the proteins (Figure 3 and Figure 4) with conventional hydrogen bond and carbon-hydrogen bonds.

The molecular docking findings obtained in the present study can be interpreted in relation to the established role of the cyclooxygenase enzymes COX-1 and COX-2 in inflammatory processes. These enzymes catalyze the conversion of arachidonic acid into prostaglandins, which are key mediators responsible for the development of pain, inflammation, and fever (Panchal and Sabina 2023). Therefore, compounds capable of interacting with these enzymes may inhibit prostaglandin production and consequently

reduce these symptoms. Although the binding affinities of the compounds were comparatively lower than that of the reference drug Indomethacin (Figure 5), they were still able to interact with important amino acid residues within the catalytic pocket of the enzymes. Such interactions suggest a potential ability of these phytocompounds to interfere with enzyme activity. Previous reports indicate that suppression of COX activity leads to decreased production of prostaglandins, particularly PGE_2 , which plays a critical role in the mediation of inflammatory pain and fever (Ricciotti and FitzGerald, 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) act mainly through this pathway by blocking prostaglandin synthesis. Therefore, the binding interactions observed between the identified phytocompounds and the COX enzymes in the present study provide a molecular basis supporting the experimentally observed analgesic, anti-inflammatory, and antipyretic effects.

On *in-vivo* experiments, acute toxicity research was done as per Organisation for Economic Co-operation and Development (OECD) 423 guideline. After the observation for 14 days rats were free from any toxic signs and symptoms and no mortality was seen. Hence, MEAP was considered safe. Therefore, doses for *in-vivo* experiments were selected based on fractions of the maximum safe dose, where $1/10^{th}$ (200 mg/kg) was used as the low dose and $1/5^{th}$ (400 mg/kg) as the high dose. The high dose (400 mg/kg) of MEAP showed considerably significant analgesic (Figure 6 and Figure 7), anti-inflammatory (Figure 8), and antipyretic activities (Figure 9) in the experimental rats possibly due to the inhibition of synthesis of inflammatory prostaglandins in the arachidonic acid pathway by blocking both COX-1 enzyme and COX-2 enzyme.

Thus, from the *in-silico* and *in-vivo* studies, as demonstrated in Figure 10, MEAP can be considered as non-selective inhibitors of COX-1 and COX-2 with promising anti-inflammatory effectiveness by the suppression of inflammatory prostaglandins.

CONCLUSIONS

The findings suggest that methanol extract of roots of *Alternanthera philoxeroides* (Mart.) Griseb. comprises several important phytoconstituents "2,3-Dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one" and "2-Amino-5-[(2-carboxy) vinyl]-imidazole". 400 mg/kg MEAP has significant and promising analgesic, anti-inflammatory and antipyretic activities by the suppression COX-1 enzymes and COX-2 enzymes. Thus, the present study is believed to add considerable amount of scientific facts as a strong literature for assisting future researchers in isolating the active therapeutic compounds for development of drugs with minimal side effects.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance; **COX:** Cyclooxygenase; **GC**–MS: Gas Chromatography-Mass Spectrometry; **IAEC**: Institutional Animal Ethics Committee; **IL-1**: Interleukin-1;
MEAP: Methanolic Root Extract of *Alternanthera philoxeroides;*
NSAIDs: Non-Steroidal Anti-inflammatory Drugs;
OECD: Organisation for Economic Co-operation and Development;
PDB: Protein Data Bank; **PGE_2:** Prostaglandin E_2; **PGI_2:** Prostacyclin (Prostaglandin I_2);
TFC: Total Flavonoid Content; TNF-α: Tumor Necrosis Factor-alpha
TPC: Total Phenolic Content; **TXA_2:** Thromboxane A_2; **UV:** Ultraviolet

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper.

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Table 1: Phytochemical screening of extracts of roots of MEAP.

Sl. No.	Phytoconstituents	MEAP
1	Alkaloids	-
2	Glycosides	+
3	Carbohydrates	+
4	Fixed oils and Fats	+
5	Terpenoids	+
6	Tannins & phenolic compounds	+
7	Proteins	-
8	Flavonoides	+

Note: "+" represents Present, "-" represents Absent of phytoconstituents.

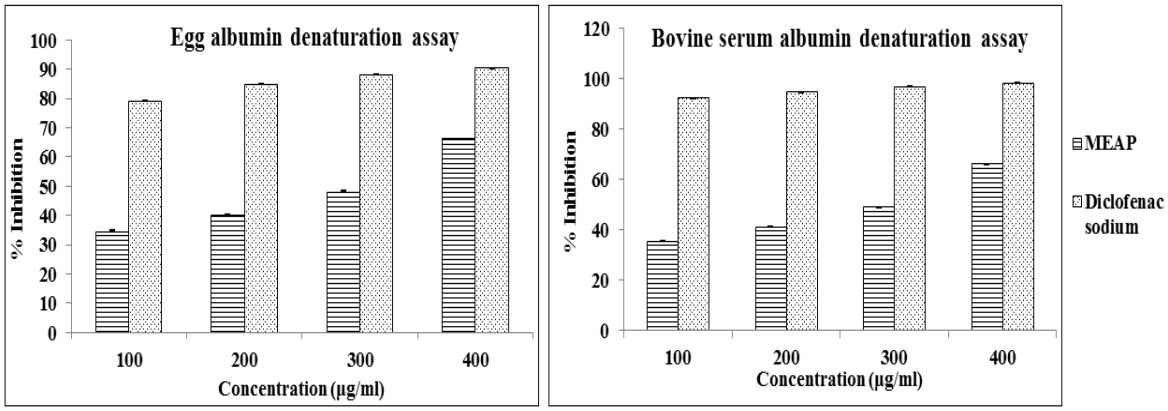

Figure 1: *In-vitro* potentiality of MEAP against inflammation by albumin denaturation assays of Egg and Bovine serum.

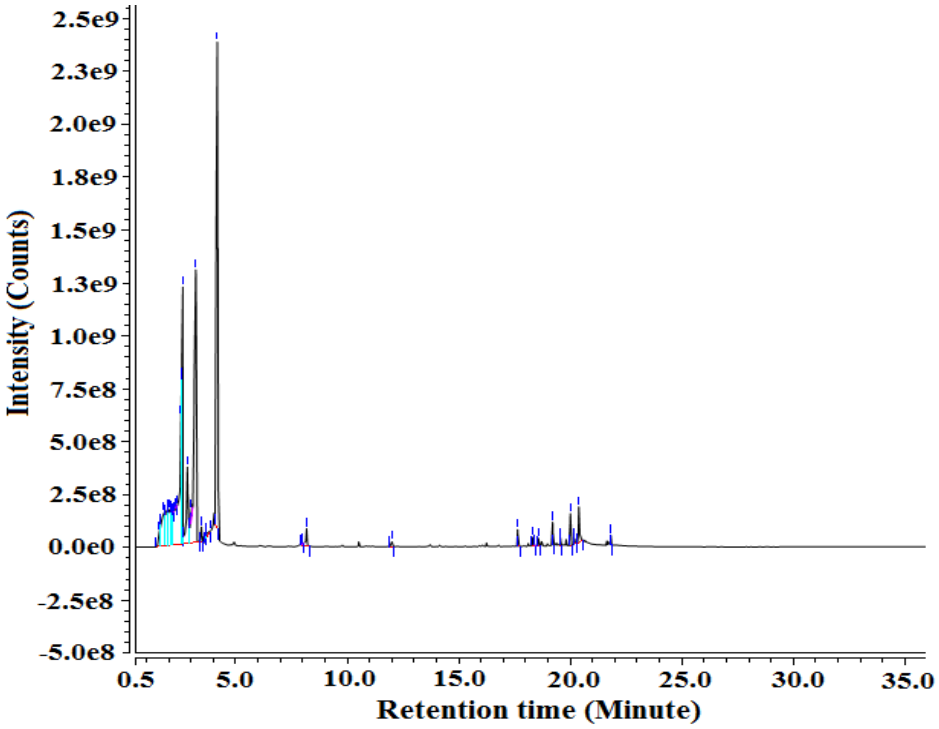

Figure 2: GC-MS chromatogram of MEAP.

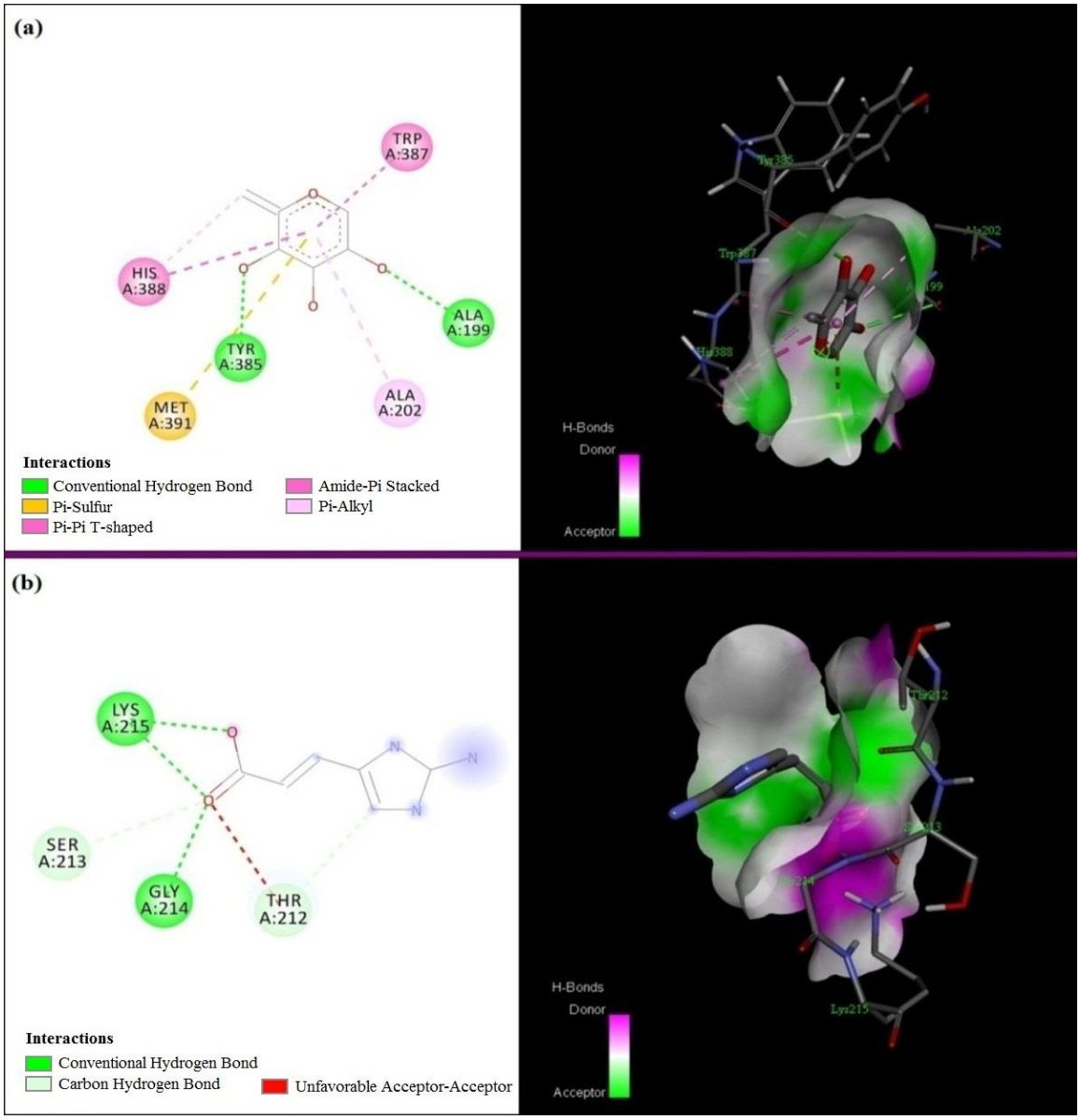

Figure 3: Molecular docking of compounds of MEAP against COX-1. (a) Docking of "2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one" against COX-1; (b) Docking of "2-Amino-5-[(2-carboxy)vinyl]-imidazole" against COX-1.

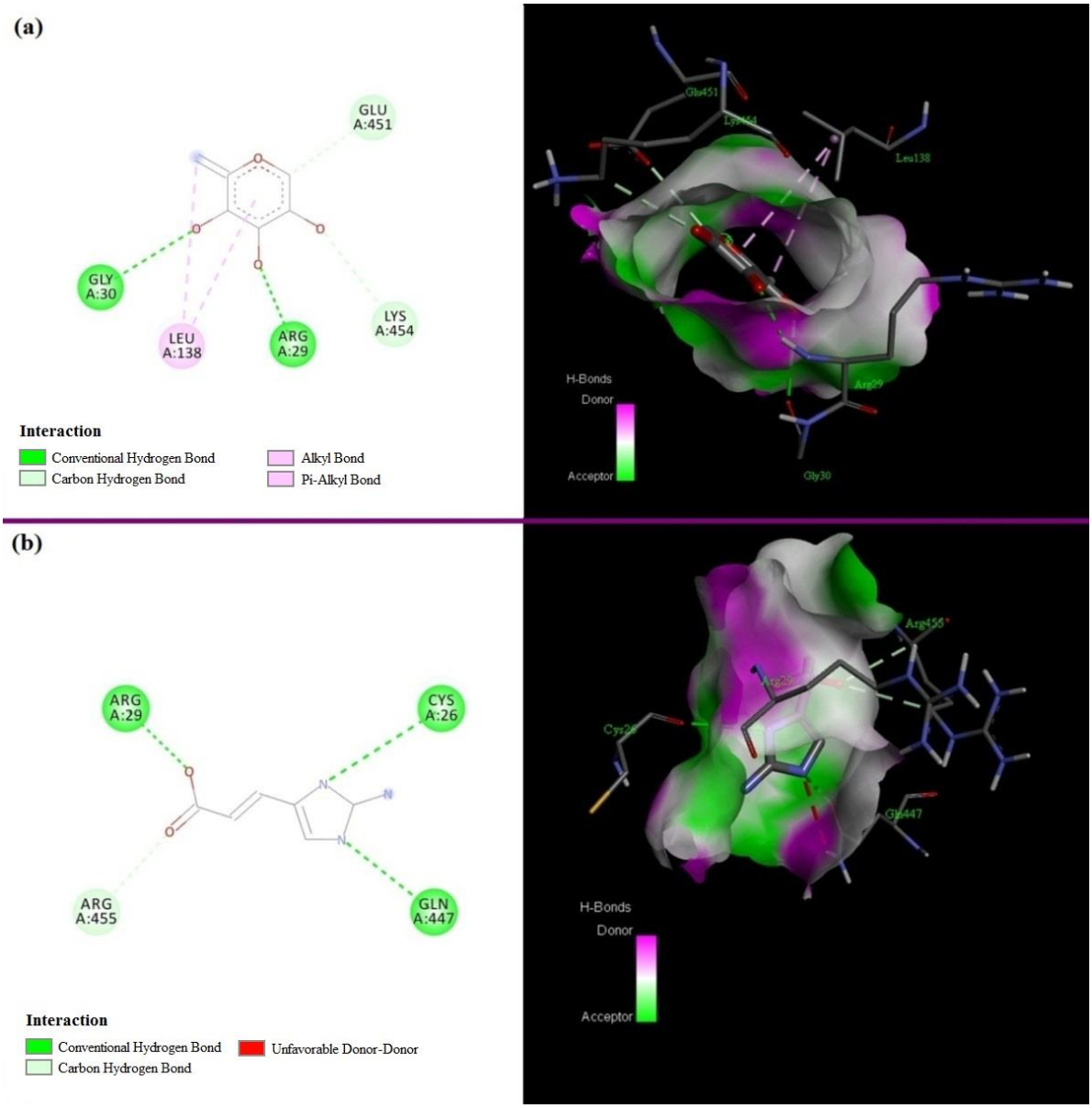

Figure 4: Molecular docking of compounds of MEAP against COX-2. (a) Docking of "2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one" against COX-2; (b) Docking of "2-Amino-5-[(2-carboxy)vinyl]-imidazole" against COX-2.

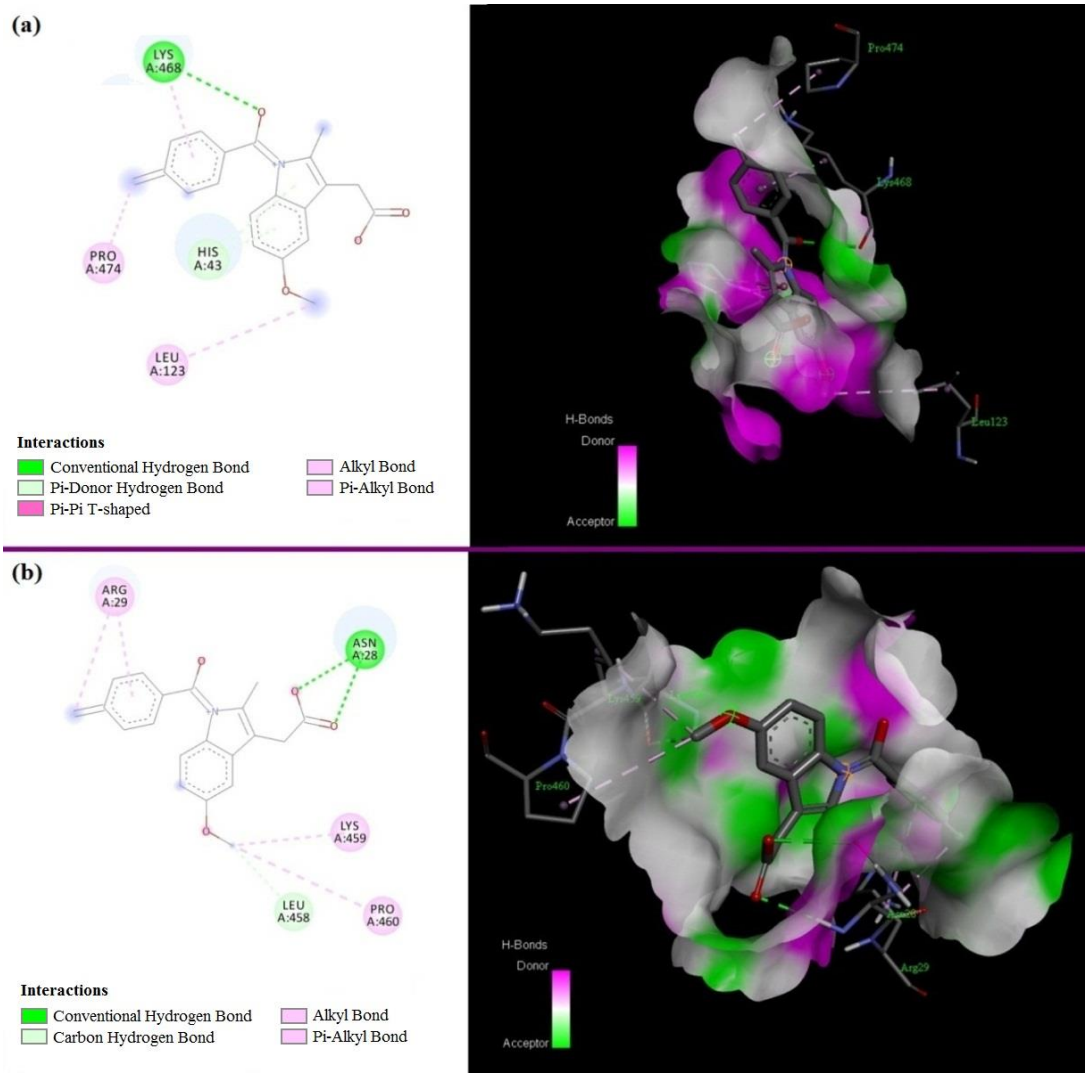

Figure 5: Molecular docking of standard drug Indomethacin against COX-1 and COX-2. (a) Docking of Indomethacin against COX-1; (b) Docking of Indomethacin against COX-2.

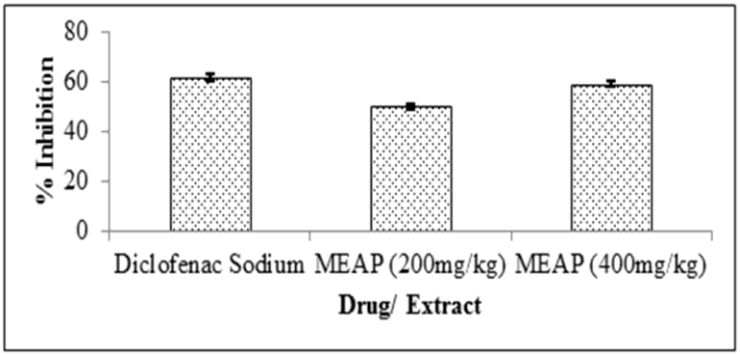

Figure 6: %Inhibition of analgesia by Diclofenac sodium and MEAP at120 minutes.

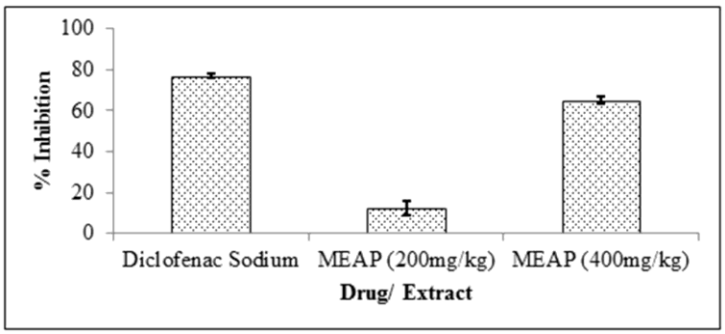

Figure 7: %Inhibition of analgesia by Diclofenac sodium and MEAP.

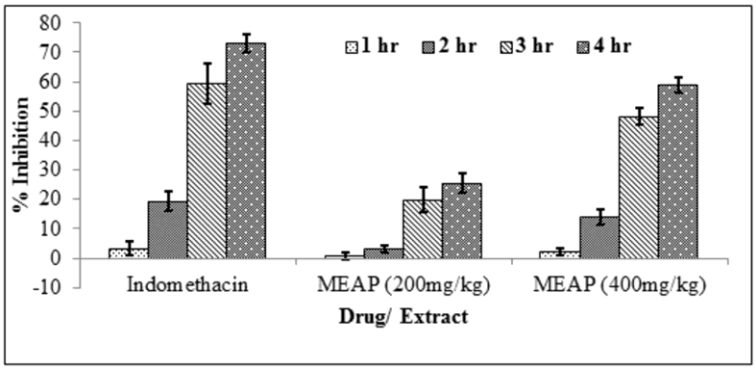

Figure 8: Anti-inflammatory activity of MEAP in experimental. Data presented as mean ± standard deviation of six variables.

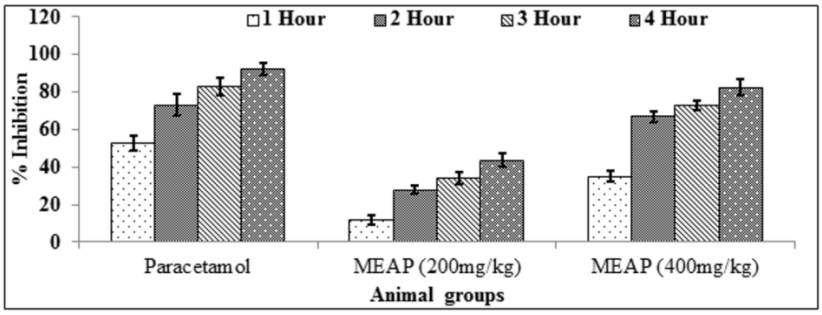

Figure 9: Antipyretic effect of MEAP in experimental rats by yeast induced pyrexia method. Data presented as mean ± standard deviation of six variables.

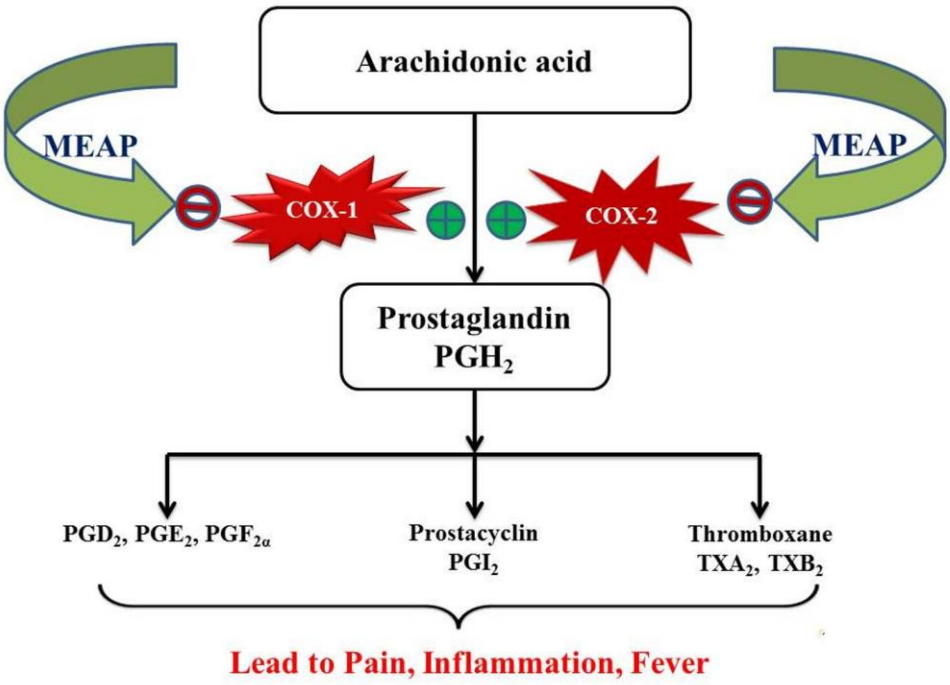

Figure 10: Effect of MEAP in the Arachidonic acid pathway. MEAP represents methanol extract of roots of *Alternanthera philoxeroides* (Mart.) Griseb. which inhibits both COX-1 and COX-2.