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Research article

Phenolic compounds and extracts from *Crotalaria calycina* Schrank potentially alleviate pain and inflammation through inhibition of cyclooxygenase-2: An in vivo and molecular dynamics studies

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HIGHLIGHTS

- Phenolic compounds were identified and quantified from MECCS via HPLC-DAD analysis.
- MECCS produced profound analgesic and anti-inflammatory activity in in-vivo models.
- Epicatechin, caffeic acid, and kaempferol showed great results in in-silico studies.

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ABSTRACT

Crotalaria calycina Schrank is a local Bangladeshi plant well-accepted by the tribal population for its medicinal properties. The primary approach of our study was to uncover the analgesic and anti-inflammatory potential of methanol extract of *C. calycina* stem in mice model with in silico molecular docking and molecular dynamics simulation approach. Phenolic compounds were identified and quantified from the extract through high-performance liquid chromatography-diode array detector (HPLC-DAD) analysis. Writhing assay through injection of acetic acid, licking assay through formalin injection, and finally, hot plate assay was employed to observe the analgesic activity. The carrageenan-induced paw edema model was employed to determine the anti-inflammatory potential of the extract. In silico molecular docking and molecular dynamics were also run to validate the in vivo study results. Eight polyphenolic compounds from the extract were identified and quantified via HPLC-DAD analysis, and (-) epicatechin was most abundantly distributed (87.15 $\pm$ 0.24 mg/100 g dry extract). In vivo study revealed that 400 mg/kg dose significantly inhibited ($P < 0.01$) the writhing response in the writhing assay and demonstrated the highest percent of inhibition of licking (70.67%) in the late part of the licking test. The same extract dose produced the highest (74.71%) percent of maximal effect (% MPE) in the hot plate assay. It demonstrated the highest percent of edema inhibition (68.00%) in the fourth hour of the paw edema assay. Molecular docking and molecular dynamics simulation of (-) epicatechin, caffeic acid, and kaempferol with cyclooxygenase-2 revealed that they have similar interactions to the standard inhibitor celecoxib. These valuable bioactive compounds may induce significant analgesic and anti-inflammatory properties in MECCS. Therefore, based on the findings of this study, it can be concluded that *C. calycina* stem can be a prospect in the medicinal field due to its remarkable analgesic and anti-inflammatory effect.

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1. Introduction

Pathological and physiological pain is described as complex, displeasing neurological events related to potential tissue injury and the release of various pain mediators. Pain is considered one of the most experienced physiological issues all around the globe and is the most common condition for which people seek medical support [1, 2]. Inflammatory effects in both PNS (peripheral nervous system) and CNS (central nervous system) are correlated with the persistence and development of several pathological pains [3]. At present, various selective and non-selective NSAIDs or non-steroidal anti-inflammatory drugs are employed to manage various inflammatory and pain-related conditions. The analgesic and anti-inflammatory principles of these drugs are to reduce the amount of arachidonic acid-driven prostaglandin (PG) and thromboxane (TX) biosynthesis that is mediated by cyclooxygenases (Coxs) [4, 5]. The generation of several biological inflammatory modulators, including as TXs, PGs, prostacyclins etc., causes the body to experience hyperthermia, inflammation, and pain is stimulated when these Cox enzymes are triggered [6]. Cox-1, one of the two principal isoforms of the Cox enzyme (the other being Cox-2), is constitutively distributed in the majority of mammalian tissues and cells. Although Cox-2 expression is minimal under normal circumstances, tissue injury and the inflammatory response of various pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, and interferon-γ cause the activation and production of Cox-2 protein [7, 8]. However, inhibiting the "housekeeping" Cox-1 enzyme interferes with normal renal function, disrupts the normal gastric mucosa defense system, and causes platelet aggregation [9, 10]. As a result, long-term or chronic use of non-specific NSAIDs (inhibitors of Cox-1) is linked to serious gastrointestinal problems, such as bleeding and ulcers, and in severe cases, liver damage and renal impairment [9, 11]. Chronic consumption of these drugs may cause serious medical conditions. For instance, patients diagnosed with liver cirrhosis may run the risk of renal failure and irregular bleeding episodes because less PG-mediated blood flow goes to the kidneys, even though healthy people generally experience little to no side effects from taking these drugs [12]. Therefore, the best method for treating inflammatory and pain diseases is through the use of selective Cox-2 inhibitors. After receiving initial approval, a number of selective Cox-2 inhibitors were driven off the market due to their high toxicity index and negative cardiovascular consequences. The fundamental goal of the research is to find a lead of selective Cox-2 inhibitors that will work without interfering with the function of Cox-1 and would be the best treatment strategy for inflammatory disorders.

The crotalaria genus, also commonly acquainted as rattlebox, is a genus of flowering plants under the Fabaceae family (legume), consisting of more than 700 species of various shrubs and herbaceous plants [13]. Although several species of crotalaria are poisonous (especially roots and seeds), various species of crotalaria are consumed as food in different parts of the world. For example, in northeast India, *C. tetragona* is consumed as a vegetable. In East Africa, *C. brevidens* is harvested considering its nutritional value (as it is a rich source of β-carotene) [14, 15]. Various chromatographic techniques are available to separate, identify and determine the phenolic composition of medicinal plant extracts. Among them, HPLC-DAD analysis is the most simple, reliable, and feasible technique widely employed for phenolic compounds analysis [16]. Phenolics or phenolic compounds are secondary plant metabolites and are the fundamental compounds of medicinal plants which can be synthesized in the laboratory through the shikimic acid and phenylpropanoid pathways [17, 18]. Several studies reported that these phytochemicals involve many pharmacological actions, including antioxidants, analgesic, anti-inflammatory, antipyretic, anti-arthritis, antimicrobial, hepatoprotective, immunomodulatory, anti-thrombotic, vasodilatory activities, and represent them as potential candidates for pharmaceutical and medicinal applications [19, 20, 21, 22]. However, various phenolics and other bioactive compounds, including myricetin, quercetin, rutin, p-hydroxybenzoic acid, phthalic acid, triterpenoid saponins, triterpenoid saponins are isolated from different species of crotalaria [23, 24]. Our investigating plant was *C. calycina* which remains in pristine condition; therefore, an investigation is needed to uncover its pharmacological potential. Therefore, our present experiment was designed to explore the analgesic and anti-inflammatory potentiality of methanol extract of *C. calycina* stem. Additionally, the phenolic compounds in the extract were identified using HPLC-DAD analysis and in silico computational analysis, i.e., the molecular docking and molecular dynamics study of the identified compounds have also been directed to corroborate the in vivo investigation's conclusions.

2. Materials and methods

2.1. Drugs and chemicals

Diclofenac sodium was purchased from Square Pharmaceuticals Ltd., Dhaka, Bangladesh. Ketorolac and indomethacin were collected from Renata Limited, Dhaka, Bangladesh, and Opsonin Pharma Limited, Dhaka, Bangladesh. Methanol was imported from Merck KGaA, Darmstadt, Germany. Loba Chemie, India, supplied acetic acid and 37% formaldehyde solution. Orion Infusions Limited, Dhaka, Bangladesh, provided a normal saline solution (0.9% NaCl).

2.2. Plant collection

The whole plant of *C. calycina* was collected from the Sitakunda hill tract, Chattagram, Bangladesh, during the growing and flowering period. The collected plant was sent to Bangladesh National Herbarium, and Dr. Sarder Nasir Uddin, Principal Scientific Officer, identified and confirmed the plant species. A voucher sample of the plant species is conserved and deposited in the herbarium of Chittagong University for further urgent necessity (Accession number: DACB 66125).

2.3. Preparation of extract

Stem was cut apart from the whole plant and washed thoroughly. Fresh stems were dried in a shady place at room temperature for 14 days and ground into fine powder. After that, in a cleaned regular-bottomed glass reservoir, 700 gm of fine powder was plunged into 3000 ml of 80% methanol for almost 15 days with episodic stirring and jerking [25]. The resulting solution was filtered using a filter cloth and Whatman Grade 1 Qualitative Filter Papers before evaporating and concentrated

Table 1. List of identified polyphenolic compounds from MECCS via HPLC-DAD analysis.

Name of Standard	*Crotalaria calycina* (stem) (mg/100 g dry extract)
Gallic acid	Nd
3,4 dihyrdoxy benzoic acid	Nd
(-) Epicatechin	87.15 ± 0.24
Caffeic acid	51.67 ± 0.44
Catechin hydrate	Nd
Catechol	Nd
p-Coumaric acid	1.79 ± 0.03
Myricetin	2.30 ± 0.09
Vanillic acid	15.01 ± 0.15
Syringic acid	Nd
Rutin hydrate	19.92 ± 0.38
Trans-Cinnamic acid	Nd
Trans-Ferulic acid	9.22 ± 0.18
Quercetin	Nd
Rosmarinic acid	Nd
Kaempferol	2.06 ± 0.10

Nd. = Not detected.

Table 2. Limit of Detection (LOD) and Limit of Quantification (LOQ) of identified polyphenolic compounds.

Standard Compounds	Limit of Detection (LOD) (ppm)	Limit of Quantification (LOQ) (ppm)
(-) Epicatechin	0.15	0.44
Caffeic acid	0.07	0.20
p-Coumaric acid	0.06	0.19
Myricetin	0.10	0.30
Vanillic acid	0.09	0.28
Rutin hydrate	0.07	0.23
Trans-Ferulic acid	0.09	0.26
Kaempferol	0.11	0.33

using a rotary evaporator. This generated a greenish sticky concentrate which was sealed in a clean glass vial and preserved at 4 degrees Celsius in a refrigerator as crude methanol extract for further subsequent investigations.

2.4. Preparation of sample and concentration

This study determined the doses based on the median lethal dose (LD50) in an acute toxicity study. To prepare and administer the extract at a dose of 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of mice, a mother solution was prepared by mixing 100 mg dry extract with 10 ml distilled water. After homogenous mixing, different doses of the extract were prepared and administered, considering the body weight of the mice. Doses of standards including diclofenac (10 mg/kg) for writhing assay and paw licking assay, ketorolac (10 mg/kg) for hot plate assay, and indomethacin (10 mg/kg) for paw edema assay were also prepared as the same procedure of extract dose preparation [26]. The dose of standards (10 mg/kg) was determined from the previous studies where 10 mg/kg of diclofenac, ketorolac, and indomethacin were used to determine the analgesic and anti-inflammatory effect of extract [25, 27].

2.5. HPLC-DAD analysis

Using a Shimadzu LC-20A (Tokyo, Japan) instrument, the methanol extract of *C. calycina* stem was analyzed to determine the phenolic profile of the extract. The apparatus included a C18 Phenomenex Luna 5 m liquid chromatography column (250 4.6 mm), a degasser (DGU-20A5), a diode array detector (SPD-20A), and a heating furnace (CTO-20A) for the column. For the chromatographic separation of phenolic chemicals, the method used two different mobile phase solutions. Preparation of solution A and solution B and the subsequent setting used to identify the phenolic metabolites in MECCS were done following Talukder et al. 2022 [28]. 0.5 mL/min flow rate and 20 μL injection volume were maintained throughout the analysis. By evaluating the obtained UV spectrum and retention time and contrasting them with the reference standard, phenolic metabolites were identified. The concentration of each compound was determined based on a peak area under 254 nm, and the results were reported as mg per 100 gm dry MECCS.

Table 3. Effect of MECCS on the number of writhing responses in acetic acid induced writhing assay.

Treatment	Number of writhing	% of inhibition
Control (DW)	81.67 ± 7.24	-
Diclofenac (10 mg/kg)	28.17 ± 4.26***	65.51
MECCS (100 mg/kg)	66.67 ± 7.70	18.37
MECCS (200 mg/kg)	46.17 ± 9.08*	43.47
MECCS (400 mg/kg)	33.5 ± 10.96**	58.98

Values are expressed as Mean ± S.E.M (n = 6). $P < 0.0001$ (****), $P < 0.001$ (***), $P < 0.01$ (**) and $P < 0.05$ (*) as compared to control (One-way ANOVA followed by post hoc Bonferroni's test).

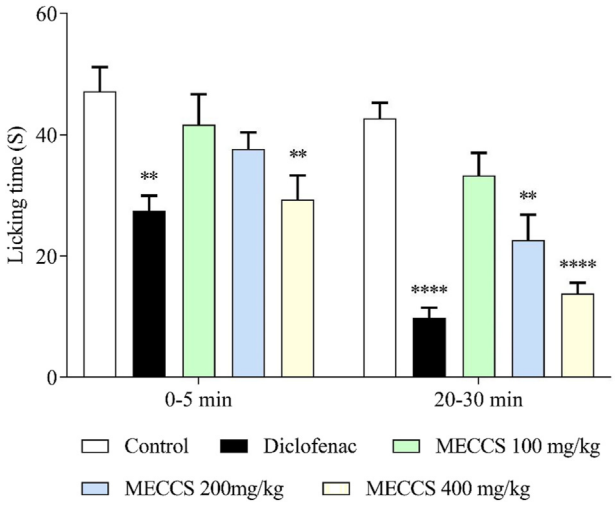

Figure 1. Effect of MECCS on licking response in formalin-induced paw licking test. Values are expressed as the Mean ± SEM (n = 6) and all data were analyzed using two-way ANOVA followed by post hoc Bonferroni test for multiple comparisons for means where level of significance are $P < 0.0001$ (****), $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*) as compared to control.

2.6. Experimental animals and ethical statement

The animal house of Jahangirnagar University, Dhaka, Bangladesh, supplied healthy Swiss-albino mice (20–25 gm) of either sex to conduct the experiment. To acclimatize the experimental mice to standard laboratory conditions, they were preserved in a facilized plastic lockup (40 cm × 30 cm × 17 cm) at a laboratory having an environment with natural illumination and half/half dark-light cycle. During the experimental time, the mice were nursing with ad libitum food and water. The Dedicated Animal Ethics Committee of Noakhali Science and Technology University rigorously reviewed all animal ethics, including protocols and procedures of the experiments, and provided approval to conduct the research (No. 61/2021).

2.7. Analgesic activity assay

2.7.1. Acetic acid-induced writhing assay (AWA)

For the evaluation of in vivo analgesic potential of MECCS, AWA was employed following the earlier described process with slight alterations [29]. Thirty healthy mice of both sexes were separated into five groups, with six mice in each group to lead the test. The experimental mice were kept in fast condition for 18 h before the experiment. The mice of each experimental group received treatment as follows; G-I (control group): experimental animals were supplied 20 ml/kg distilled water; G-II (standard group): all experimental mice were treated with 10 mg/kg diclofenac; animals of G-III, G-IV, and G-V were treated with 100, 200, and 400 mg/kg of MECCS, respectively. All mice received an intraperitoneal injection of acetic acid solution (0.6%, 0.1mL/10g) to initiate the recognizable writhing after 30 min of receiving the appropriate therapy. The appropriate absorption was confirmed one more with 5 min of interspace, and then 15 min were spent counting the writhing numbers.

Table 4. Effect of MECCS on percent inhibition in hind paw licking test model.

Treatment	Percent inhibition of licking (PIL)	
	Early phase (0–5 min)	Late phase (20–30 min)
Diclofenac (10 mg/kg)	41.69	79.15
MECCS (100 mg/kg)	11.54	29.33
MECCS (200 mg/kg)	20.21	53.00
MECCS (400 mg/kg)	37.82	70.67

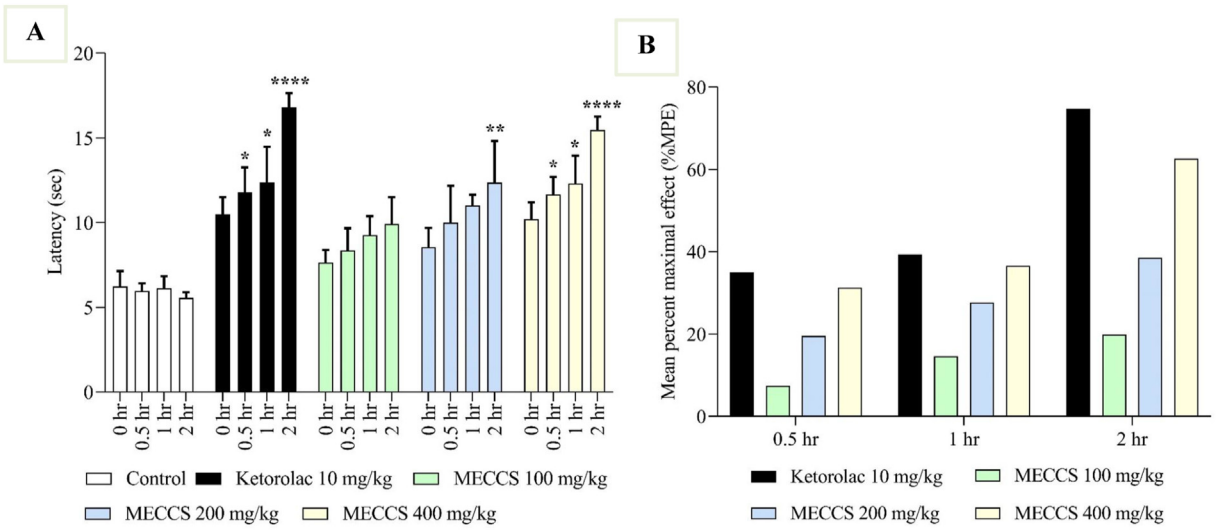

Figure 2. Effect of MECCS on latency in hot plate test. Values are expressed as the Mean ± SEM (n = 6) and all data were analyzed using two-way ANOVA followed by post hoc Bonferroni test for multiple comparisons for means where level of significance are P < 0.0001 (****), P < 0.001 (***), P < 0.01 (**), and P < 0.05 (*) as compared to control (A). Mean percent maximal effect (% MPE) of different doses of extract and ketorolac in hot plate test (B).

2.7.2. Formalin-induced paw licking assay (PLA)

This assay was performed to discern the analgesic activity of the MECCS as the procedure explained by Dubuisson and Dennis, with some modifications [30]. The arrangement of the test animals into groups and their method of treatment were similar to AWA. All mice subcutaneously received 0.02 mL of 2.5% formalin in the rear of the left hind paw following 1 h of receiving respective doses. The activity of biting and licking for the first 5 min was considered an early response. After that, the biting and licking time of the experimental mice was read during the last 20–30 min after administration as the late response. The findings of this experiment were expressed in the form of percent inhibition of licking response (PIL).

2.7.3. Hot plate assay (HPA)

This assay was done using a hot-plate analgesiometer (Orchid, India) apparatus where the plate's temperature was kept near 55 ± 0.5 °C. The experimental animals were first placed in the chamber of the apparatus, and those who demonstrated nociception within 10 s were selected for the ultimate experimental procedure. The group distribution and treatment strategy were almost identical in the HPA, like AWA and PLA. However, this assay employed ketorolac as standard with a 10 mg/kg dose. Each mouse from each group was kept on the heated surface of the hot plate device after oral administration of all therapies, and the latency to heat stimuli was assessed at 0, 0.5, 1, and 2 h later of the treatment. The time was recorded as the response time when the experimental animal first licked its paws, began to hop, or made an attempt to run away from the hot plate device. Twenty seconds cut-off time was fixed to eliminate the mice's tissue damage risk. The final findings of this assay were demonstrated as the percentage of the maximal effect (% MPE) [25].

2.8. Carrageenan-induced paw edema assay (PEA)

In this assay, 0.1 ml of freshly prepared 1% (w/v) carrageenan solution (prepared by mixing in 0.9% NaCl solution) was injected in the sub-plantar region of the right hind paw of all the test animals to cause an immediate inflammatory response [31]. One hour before the injection of carrageenan solution, all the test animals were premedicated with their respective treatment, i.e. 20 ml/kg distilled water (control group), 10 mg/kg indomethacin (standard group), and 100, 200, and 400 mg/kg MECCS (extract group). Utilizing a Vernier Caliper (SMEC, Shanghai, China), the linear paw circumference was meticulously measured at intervals of 0, 1, 2, 3, and 4 h. The findings of this experiment were

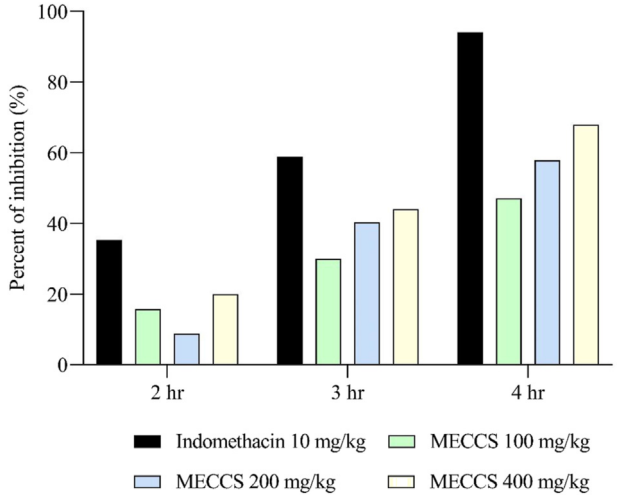

Figure 3. The percentage of inhibition of paw edema by different doses of MECCS and standard indomethacin.

Table 5. In vivo anti-inflammatory activity of different dose of MECCS extract against carrageenan induced paw edema.

Treatment	0 hr	1 hr	2 hr	3 hr	4 hr
Control	2.57 ± 0.05	3.57 ± 0.13	3.62 ± 0.06	3.73 ± 0.05	3.77 ± 0.06
Indomethacin (10 mg/kg)	2.58 ± 0.03	2.92 ± 0.09****	2.80 ± 0.07****	2.72 ± 0.08****	2.60 ± 0.05****
MECCS (100 mg/kg)	2.53 ± 0.03	3.23 ± 0.11*	3.12 ± 0.12***	3.02 ± 0.13****	2.90 ± 0.11****
MECCS (200 mg/kg)	2.58 ± 0.05	3.15 ± 0.06**	3.10 ± 0.10****	2.92 ± 0.08****	2.82 ± 0.09****
MECCS (400 mg/kg)	2.57 ± 0.02	3.07 ± 0.05***	2.97 ± 0.04****	2.85 ± 0.05****	2.73 ± 0.07****

Values are expressed as the Mean ± SEM (n = 6) and all data were analyzed using two-way ANOVA followed by post hoc Bonferroni test for multiple comparisons for means where level of significance are P < 0.0001 (****), P < 0.001 (***), P < 0.01 (**), and P < 0.05 (*) as compared to control.

calculated as a percentage of edema inhibition which was determined in accordance with the following equation,

$$\% \text{ of inhibition} = [(T_a - T_b)/(T_a - T_t)] \times 100$$

Where T_a represents the edema thickness after carrageenan injection, T_b is the edema thickness before carrageenan injection, and T_t represents the edema thickness at different time intervals.

2.9. In silico analysis

2.9.1. Molecular docking

Celecoxib's co-crystallized crystal structures with Cox-2 (PDB ID: 3LN1) were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). Elimination of various heteroatoms and water molecules were performed using PyMol software (version 2.4.1). Utilizing SPDBV software (version 4.1.0), the protein-energy reduction was accomplished, and the proteins' structures were modified by adding polar hydrogens and merging non-polar hydrogens using Auto Dock software package (Version 1.5.6) [32]. Following the addition of total Kollman charges (11.031), the protein's active site was created using a grid box with the coordinates X: -41.281, Y: 7.542, and Z: 23.021. Dimensions of the grid box was X: 20, Y: 20, and Z: 20, with an exhaustiveness of 8. Following that, pdbqt formats of the protein structure were downloaded for the docking investigations.

Three dimensional (3D) structures of identified compounds from MECCS, including (-) epicatechin (PubChem CID: 72276), caffeic acid (PubChem CID: 689043), p-Coumaric acid (PubChem CID: 637542), myricetin (PubChem CID: 5281672), vanillic acid (PubChem CID: 8468), rutin hydrate (PubChem CID: 45479757), trans-Ferulic acid (PubChem CID: 445858) and kaempferol (PubChem CID: 5280863) were retrieved in sdf formats from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and using PyMOL (version 2.4.1) converted them into pdb formats. Employing Auto Dock tools, ligands pdf files were further subsequently transformed to pdbqt formats. The ligands' energy was optimized by applying the AMBER 14 force field, and the UCSF Chimera software program. Finally, Autodock Vina (version 1.2.0) was used to accomplish docking. The optimal docking poses require molecular dynamics simulation; therefore, the best docking poses of the ligands were exported in pdb format. In order to observe the docking poses, BIOVIA Discovery Studio (version 4.5.0) was used.

2.9.2. Molecular dynamics simulation

For 25ns, apo-Cox-2 and ligand-bound Cox-2 underwent molecular dynamics. Dynamics simulation was carried out using GROMACS 2021.3 simulation software. The topology of the protein was constructed using the CHARMM36 force field [33]. The complex was created by first generating the ligand topology independently, then merging it with the protein topology using the official CGenFF or CHARMM general force field server. The protein-ligand system was solvated using the TIP3P water model. Counterions were injected into the system intended to neutralize the setup. The system's overall energy usage was decreased using the 5000 steepest descent minimization steps. Position restraint topology was generated to apply restraints to the ligands. Protein and ligands were paired together for temperature coupling. Following the system's equilibration in NVT and NPT, a 25ns primary molecular dynamics simulation was run. After completing the molecular dynamics simulation, RMSD (root-mean-square deviation), the radius of gyration, RMSF (root-mean-square fluctuation), and the number of hydrogen bonds between protein and ligand were determined.

2.9.3. Principal component analysis and Gibbs free energy calculation

The molecular dynamics simulation trajectory is used to gather dominating modes of molecule motion through principal component analysis. These atomic group motions are eliminated by superimposing a least-square fit onto the reference structure, and they are then recreated using liner conversion in Cartesian coordinates to produce a covariance matrix. The covariance matrix is diagonalized to provide an eigenvector and accompanying eigenvalues that indicate the unique energy contribution of each component. Gibbs free energy measures a protein-ligand complex's thermodynamic properties. The gmx covar command was used to calculate the covariance matrix of coordinates for alpha carbon. The coordinates were diagonalized so that the eigenvectors and eigenvalues could be more easily understood. To visualize the first two eigenvalue components, PC1 and PC2, the gmx anaeig program was used. The Gibbs free energy landscape was determined using the gmx sham command. Using Origin 2021b, a free energy landscape plot was generated.

3. Statistical analysis

All data were presented as Mean ± SEM (standard error mean), and all statistical analysis was conducted with the utilization of GraphPad Prism (version 8.0.2). Depending on the assay, one-way or two-way analysis of variance or ANOVA followed by post hoc Bonferroni test was employed to describe the statistical significance. P values of $P < 0.0001$ (****), $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*) were considered as statistically significant.

4. Results

4.1. Phenolic composition

HPLC-DAD analysis identified eight phenolics from the MECCS (Table 1). (-) Epicatechin and caffeic acid were found to be distributed most abundantly in the stem extract (87.15 and 51.67 mg/100g dry extract, respectively). Rutin hydrate (19.92 mg/100g dry extract) and vanillic acid (15.01 mg/100g dry extract) were also in considerable amounts. In contrast, trans-ferulic acid (9.22 mg/100g dry extract), myricetin (2.30 mg/100g dry extract), kaempferol (2.06 mg/100g dry extract), and p-coumaric acid (1.79 mg/100g dry extract) existed in the least amount in the MECCS. Since these polyphenolic standards are commonly present in medicinal plants and available in our research Lab, we choose these 16 polyphenolic standard compounds. The validation of the method and the quantification were performed following ICH procedures (ICH, 2012). The LOD (Limit of Detection) and LOQ (Limit of Quantification) are demonstrated in Table 2.

Table 6. Number of hydrogen bonds, binding affinity and interaction of ligands with amino acid residues found after molecular docking.

Ligand	PubChem CID	No of H bond	Binding affinity (kcal/mol)	Bonding interactions (AA … Ligand)	Bond length (Å)
Celecoxib	2662	4	−8.7	ARG482(N–H···O–S) PHE487(N–H···O–S) LEU321(C–O···H–N) SER322(C–O···H–N)	2.206 2.739 2.718 2.069
(-) Epicatechin	72276	1	−7.0	MET491(C–O···H–O)	2.604
Kaempferol	5280863	4	−6.1	TRP356(N–H···O–C) SER499(O–H···O–C) SER499(O–H···O–C) GLY495(C–O···H–O)	2.522 3.043 2.224 3.035
Caffeic acid	689043	2	−6.2	TYR354(C–O···H–O) TYR324(O–H···O–C)	2.526 2.187
P-coumaric acid	637542	2	−6.1	SER499(O–H···O–C) TYR354(C–O···H–O)	2.018 2.006
Trans-ferulic acid	445858	2	−6.4	ARG89(N–H···O–C) TYR324(C–O...H–O)	2.558 2.258

4.2. Analgesic activity assay

4.2.1. Acetic acid-induced writhing assay (AWA)

Results of the AWA in which oral treatment with MECCS showed dose-dependent analgesic activity in the experimental model are demonstrated in Table 3. After injection of acetic acid into the experimental animals, MECCS 100, 200, and 400 mg/kg dose reduced the number of writhes to 66.67 ± 7.70 with 18.37% inhibition, 46.17 ± 9.08 ($P < 0.05$ and $t = 3.08$) with 43.47% inhibition, and 33.5 ± 10.96 ($P < 0.01$ and $t = 4.18$) with 58.98% inhibition, respectively, where control group's writhing was 81.67 ± 7.24. The percent of inhibition for the standard was found to be 65.51% (writhing was 28.17 ± 4.26, $P < 0.001$ and $t = 4.64$), and the writhing inhibition percent of 400 mg/kg extract dose was almost close to standard drug diclofenac.

4.2.2. Formalin-induced paw licking assay (PLA)

Oral administration of MECCS demonstrated a decrease in the paw-licking time when compared to the control group in both phases of the experiment (Figure 1). In the early experiment phase (0–5 min), diclofenac and MECCS 400 mg/kg dose significantly ($P < 0.01$, $t = 4.15$ and 3.74, respectively) reduced the licking time when compared to the control group. MECCS 100 mg/kg dose induced a poor inhibitory effect (11.54%) on licking response. However, increasing doses gradually enhanced inhibitory effects, demonstrating 20.21% and 37.82% inhibitory effects at 200 and 400 mg/kg, respectively. In the late phase (20–30 min) of the experiment, MECCS 200 mg/kg and 400 mg/kg doses significantly reduced the licking time ($P < 0.01$ and $t = 4.19$; $P < 0.0001$ and $t = 6.05$, respectively) and produced noticeable analgesic activity (53.00% and 70.67%, respectively) when compared to the standard drug diclofenac ($P < 0.0001$ and $t = 6.89$) (79.15% inhibition) (Table 4).

4.2.3. Hot plate assay (HPA)

MECCS 400 mg/kg dose significantly enhanced the latency time from 10.19 sec to 15.48 sec, $P < 0.0001$ and $t = 5.39$, comparable to the standard ketorolac group (10.51 sec–16.81 sec, $P < 0.0001$ and $t = 6.12$) (Figure 2A). This dose of the extract also produced the highest mean percent maximal effect (62.65%) after the second hour of treatment, where % MPE of standard ketorolac was (74.71%) after 2 hr of treatment (Figure 2B). On the other hand, MECCS 100 and 200 mg/kg doses also produced comparable percent of inhibition (19.88% and 38.53%, respectively) after 2 hr.

4.3. Carrageenan-induced paw edema assay (PEA)

Table 5 represents the changes in paw thickness after the administration of carrageenan and experimental extracts. Figure 3 demonstrates the results of the percentage of edema inhibition after administering standard and different doses of MECCS. MECCS 400 mg/kg dose demonstrated anti-inflammatory action (% of edema inhibition = 20.00–68.00) at various time intervals (2–4 hrs) that was comparable to standard indomethacin (% of edema inhibition = 35.29–94.12). On the other hand, MECCS 100 mg/kg dose showed weak anti-inflammatory action (% of edema inhibition = 15.71–47.14), and MECCS 200 mg/kg dose demonstrated moderate anti-inflammatory action (% of edema inhibition = 8.77–57.89) compared to the inhibition of indomethacin.

4.4. In silico analysis

4.4.1. Molecular docking

The docking of celecoxib and other MECCS compounds with Cox-2 was conducted, and the result is outlined in Table 6. The ligands showing affinity higher than -6.0 kcal/mol were considered. Five HPLC-

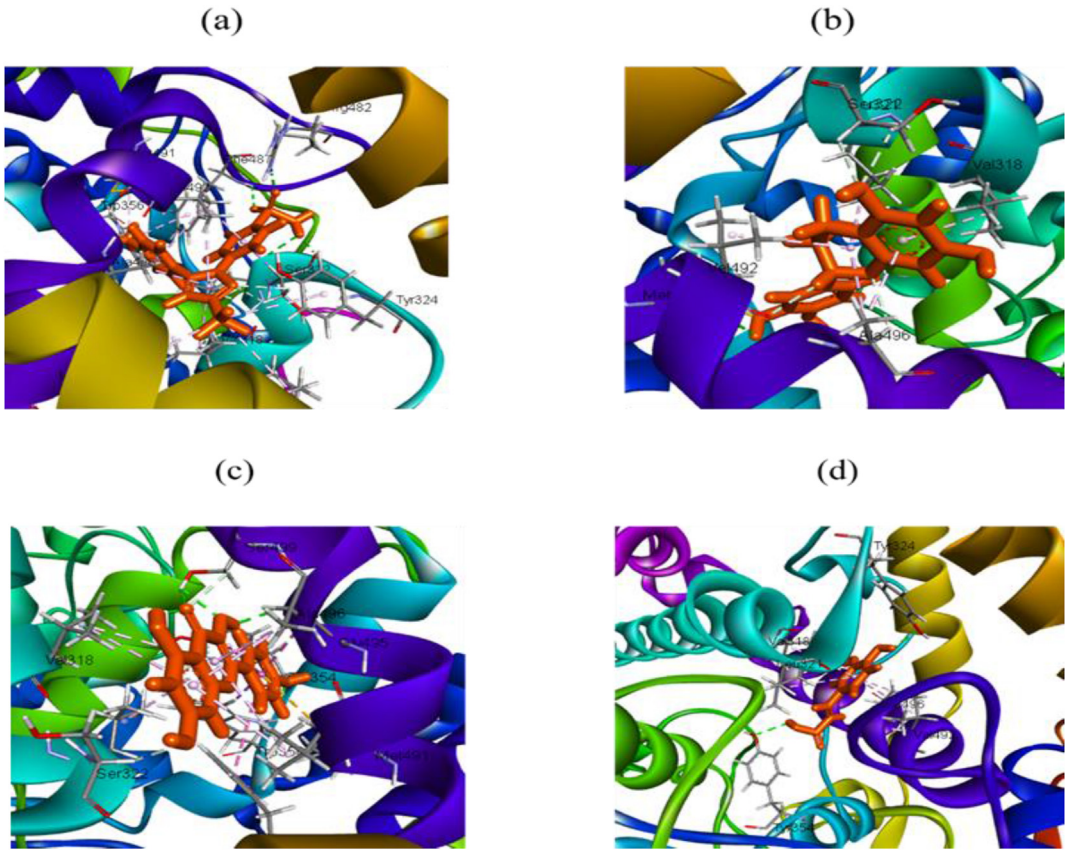

Figure 4. Non-covalent interactions of selected phytochemicals (a) celecoxib, (b) (-) epicatechin, (c) kaempferol, and (d) caffeic acid with COX-2 (PDB ID:3LN1) (Pose predicted by AutoDock Vina).

identified compounds showed binding affinity higher than −6.0 kcal/mol. The standard celecoxib showed the highest affinity (−8.7 kcal/mol). Among the MECCS compounds, (-) epicatechin is the top scorer based on affinity (−7.0 kcal/mol). Celecoxib and kaempferol have the highest hydrogen bonds formed during molecular docking analysis. The rest of the ligands showed ≥1 hydrogen bond with the binding pocket of Cox-2. In MECCS, (-) epicatechin and caffeic acid have the highest concentration (87.15 ± 0.24 and 51.67 ± 0.44 mg/100 g dry extract, respectively). They also show an excellent affinity with Cox-2. On the other hand, kaempferol has a low concentration (2.06 ± 0.10 mg/100 g dry extract), but it shows a significant number of hydrogen bonds with Cox-2 with fair, binding affinity. To validate their docking efficiencies, (-) epicatechin, caffeic acid, and kaempferol were used for subsequent molecular dynamics simulations. The 3D docking poses are also illustrated in Figure 4a, b, c, for celecoxib, epicatechin, kaempferol, and caffeic acid, respectively. The 2D interactions of these ligands are also demonstrated in Figure 5a, b, c, d, respectively.

4.4.2. Analysis of molecular dynamics simulation

25ns long dynamics simulation has been carried out for standard drug celecoxib and phytocompounds with Cox-2 (Figure 6a). Molecular dynamics run for apo Cox-2 has also been conducted. During the simulation, the apo Cox-2's RMSD for the alpha-carbon was within 0.1 nm–0.25 nm, showing higher stability. When attached to celecoxib, Cox-2 displayed RMSDs for the alpha carbon ranging from 0.1 to 0.15 nm, demonstrating a relatively stable structure. Kaempferol shows identical RMSD with celecoxib. Caffeic acid's RMSD is more similar to apo Cox-2. Though (-) epicatechin shows RMSD fluctuation at 7.5ns, and it becomes more consistent. All the apo and ligand-bound Cox-2 show better stability in terms of RMSD.

The measurement of a protein's structural compactness is its radius of gyration. The degree of protein compactness increases with decreasing fluctuations in radius of gyration. The results of all simulations displayed almost identical radius of gyration ranging from 2.4 to 2.45 nm (Figure 6b). The constancy of the radius of gyration was consistent throughout the simulation, indicating better system rigidity.

Measurements of residue specific RMSD provide insight into the regions with significant fluctuations. The total RMSF of apo and ligand-bound Cox-2 are nearly identical, as seen in Figure 6c. However, when compared to apo Cox-2, the hydrogen bonds that were generated between the residues and the ligands displayed lesser variance.

Interactive hydrogen bonds play a significant role in the stability of both inhibitors and proteins [34]. Figure 7 shows the intermolecular hydrogen bonds that Cox-2 and its ligands produced over the course of the 25ns dynamics. During the entire 25 ns, the control celecoxib produced 1–3 hydrogen bonds with Cox-2 (Figure 7a). (-) Epicatechin initially has 0–2 hydrogen bonds with Cox-2, but as the dynamics proceeds, the number of hydrogen bonds increases ranging from 2-4 (Figure 7b). Kaempferol has 1–4 hydrogen bonds during 25ns dynamics (Figure 7c). Caffeic acid has 0–2 hydrogen bonds formed with Cox-2 in the whole dynamics (Figure 7d).

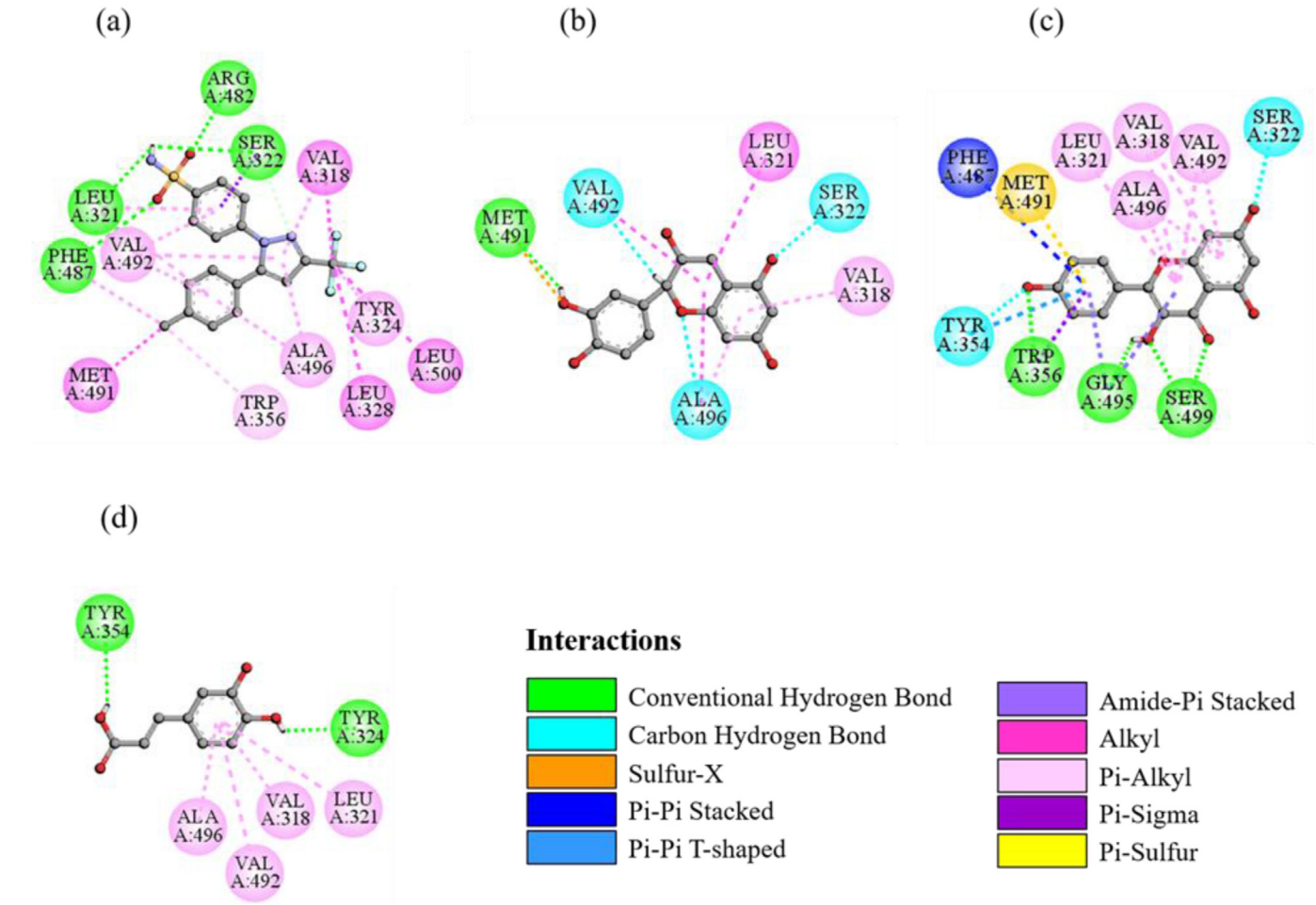

Figure 5. Non-covalent interactions of selected phytochemicals (a) celecoxib, (b) (-) epicatechin, (c) kaempferol, and (d) caffeic acid with Cox-2 (PDB ID:3LN1) (Pose predicted by AutoDock Vina).

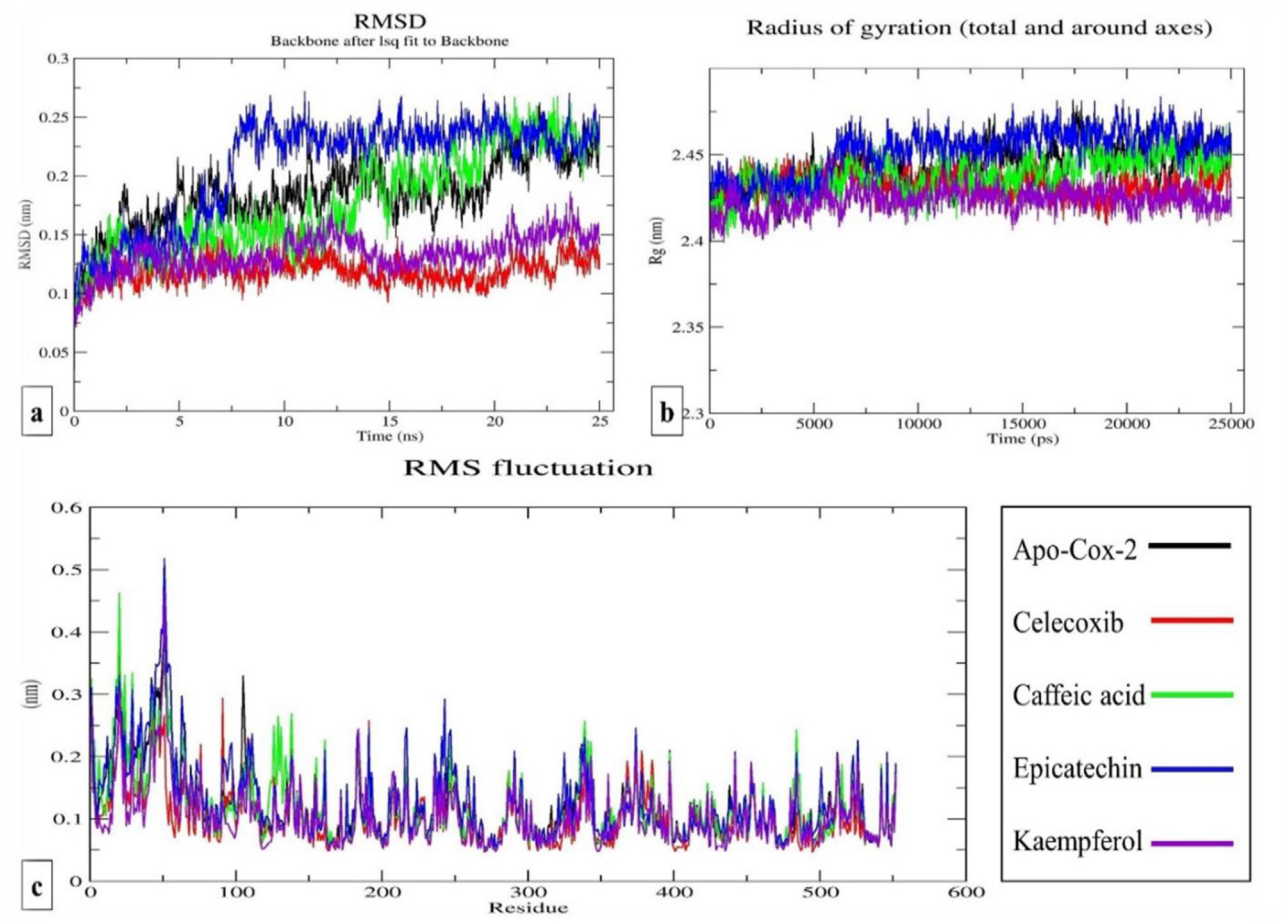

Figure 6. Results of 25ns molecular dynamics simulation of Cox-2 with ligands. (a) Root mean square deviation (RMSD) (b) Radius of gyration (c) Residual root mean square fluctuations (RMSF).

4.4.3. Principal component and free energy landscape analysis

The conformational sampling of apo Cox-2 and the ligand-Cox-2 complex has been investigated in this study using principal component analysis. The covariance matrix gathered the eigenvalues that were associated with the relevant eigenvectors. Alpha carbon superimposed the first two eigenvectors onto each other. The principle component analysis involved in calculating the Gibbs free energy landscapes (FEL). FEL is demonstrated in Figure 8a, b, c, d, e for apo-Cox-2, Cox-2 bound with celecoxib, Cox-2 bound with kaempferol, Cox-2 bound with caffeic acid, and Cox-2 bound with (-) epicatechin, respectively. Apo Cox-2's FEL showed a significant amount of deep blue colored basin containing the lower energy state. This denotes a more advantageous thermodynamic state. As we move on to the Cox-2-celecoxib complex, we notice that the internal basin barrier has vanished, leaving behind a considerable number of deep blue colored basins. However, compared to apo-Cox-2, lower peaks render lower energy states. When kaempferol is taken for analysis, it shows a similar type of FEL with apo-Cox-2. The basins contain more thermodynamically favorable states than other complexes. Caffeic acid shows significant numbers of lower energy states even though the conformational pattern is quite different from other complexes of Cox-2.

5. Discussion

Since ancient times, the tribal population has utilized plants and herbs to treat various ailments, and nature has become a credible source of therapeutics for them [35]. Nowadays, medicinal plants are coming to light and asserting their importance in isolating novel therapeutic entities [36]. Nevertheless, most medicinal plants remain pristine, and these auspicious plants have never been tested to uncover pharmacological and toxicological properties or to identify precious bioactive compounds [2]. *C. calycina* is a medicinal plant whose medicinal and pharmacological properties have not been revealed yet. We have separated eight phenolic metabolites from the stem part of this medicinal plant by employing HPLC-DAD analysis. Among them, (-) epicatechin and caffeic acid are most abundantly distributed (87.15 and 51.67 mg/100g dry extract, respectively) in the stem of this plant. The anti-inflammatory and analgesic properties of (-) epicatechin and caffeic acid have been demonstrated in a number of in-vivo studies [37, 38].

In studies to examine the possible anti-inflammatory potential of natural or drug compounds, carrageenan is frequently utilized as a pro-inflammatory agent [39]. Carrageenan injection under the skin causes edema or swelling in the paw. By monitoring the change in paw thickness over time, the ability of experimental subjects to suppress edema (% of edema inhibition) was calculated at various time intervals, say, 1, 2, 3, and 4 hr of the experiment. However, it is widely known that carrageenan-induced edema results from a biphasic mechanism. The release of serotonin, histamine, bradykinins from the mastocytes or mast cells in the injured tissue characterizes the first phase of the inflammatory reaction, typically occurring 1–2 h after carrageenan injection. Interleukins (IL-10, IL-6, and IL-1), TNF-α, and different arachidonate metabolites like leukotrienes and prostaglandins are stimulated during the second phase of the inflammatory response, which typically occurs 3–6 h after the carrageenan injection [40]. In our experiment, MECCS 400 mg/kg dose showed the highest effect in the fourth hour. So, it stands to reason that this dose of the extract likely exerted its anti-inflammatory

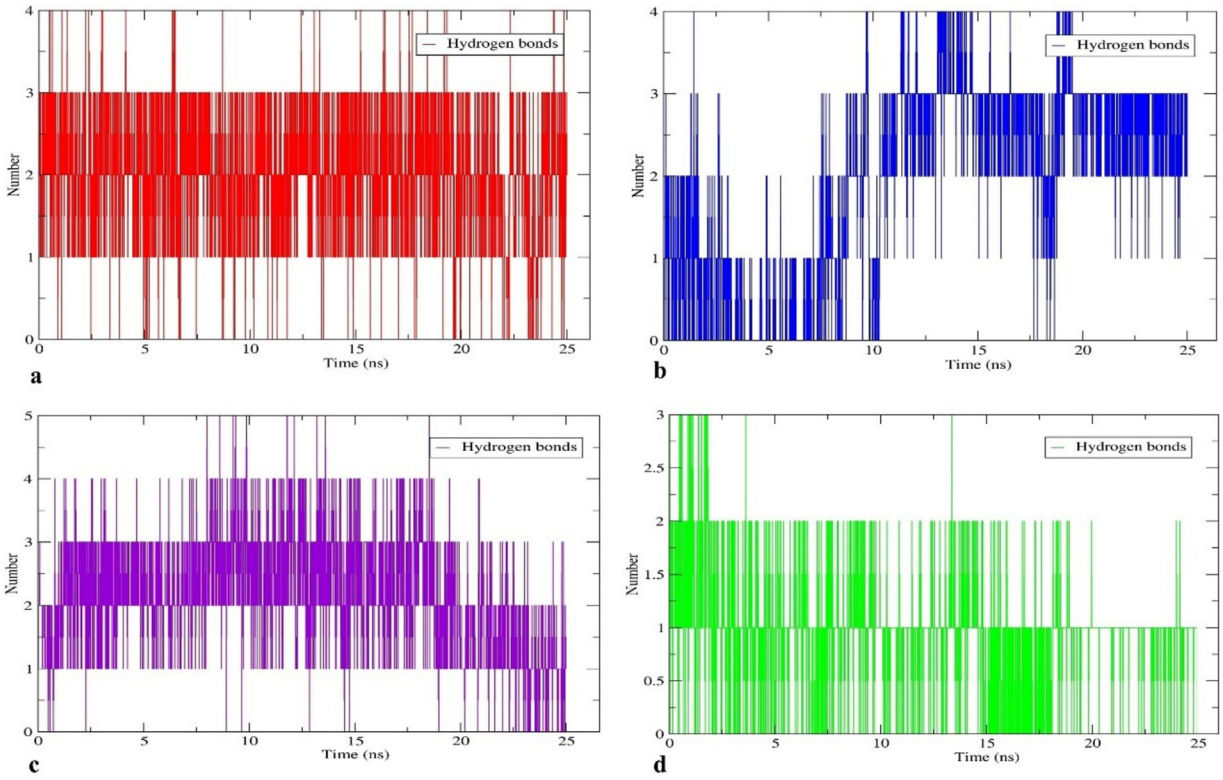

Figure 7. Average number of hydrogen bonds between Cox-2 and ligands during 25ns molecular dynamics simulation. (a) celecoxib (b) (-) epicatechin (c) kaempferol (d) caffeic acid.

effects by preventing the release of inflammatory mediators. It has been reported that carrageenan injection stimulated the release of AA and lactate dehydrogenase from rat pleural cells [41]. AA releases three primary classes of eicosanoids: prostaglandins, thromboxanes, and leukotrienes, which are actively responsible for inflammatory action. Eicosanoid signaling is similar to cytokine signaling, and inflammasome formation and suppression of the formation or interference in the receptor-mediated action of eicosanoids is the prevailing strategy to treat inflammatory disorders [42].

It is a general consideration that inflammation is strongly correlated with pathological pain, and therefore, the drugs used to treat inflammatory disorders are also assumed to have analgesic potential. AWA is one of the most acceptable non-selective experimental models to uncover the analgesic effectiveness of drugs. The administration of acetic acid induces peritoneal inflammation by activating the somatic and visceral nociceptors that innervate the peritoneum and stimulate an inflammatory response in both subcutaneous muscle walls and sub-diaphragmatic visceral organs [43]. On the other hand, acetic acid is also responsible for increasing capillary permeability. It induces pain through exciting nerve endings triggered by the release of various pain mediators, including prostaglandin, peritoneal mast cells, and so forth. Traditional NSAIDs produce analgesic and anti-inflammatory action by inhibiting Cox in peripheral tissue and Cox interference with the signal transduction of primary afferent nociceptors [25]. In our study, MECCS 400 mg/kg dose demonstrated the highest percentage of writhing inhibition (58.98%), which may be due to the disturbance of pain and inflammatory pathways. To validate the writhing test results, however, another experimental model must be used because of the non-specific nature of this investigation. To do this, a biphasic PLA was used. The intradermal injection of formalin triggers a biphasic nociceptive response, and the first phase of the response, which lasts for 0 to 5 min, is thought to be caused by the stimulation of peripheral nociceptors by formalin, which creates an acute obstruction to activity. On the contrary, the release of inflammatory cytokines, such as prostaglandins, bradykinin, 5HT, etc., into the spinal cord of peripheral nociceptors causes the establishment of an inflammatory response during the second phase (10–45 min). Drugs that block both stages are thought to cause central antinociceptive activity, whereas drugs with peripheral actions produce antinociceptive action by inhibiting the second phase [25, 44]. Since MECCS 400 mg/kg dose exhibited a maximum percent of inhibition (70.67%) in the experiment's inflammatory phase (late phase), it can be assumed that there is a possibility of inhibiting the inflammation-induced cytokines in the periphery or spinal cord. In HPA, different doses of MECCS extract produce a dose-dependent increase of reaction time with the highest inhibition after 2 hrs of the experiment. In response to thermal stimuli, the opioid μ receptor work in the frontline to regulate thermal pain, and stimulation of this receptor produce spinal analgesic activity. The increase in reaction time may be centrally mediated due to its effect on the opioid receptor [45]. Caffeic acid has been reported to generate an anti-nociceptive effect mediated by opioid receptors. Phenolics or phenolic compounds are well known for their antioxidant properties. ROS or reactive oxygen species are considered the crucial signaling molecules responsible for the progression of various inflammatory disorders [46]. ROS that are formed in biological systems are capable of reacting effectively with DNA, proteins, and lipids. Changes in cellular function can be attributed to the modification of proteins that govern regulatory and transcription factors. Changes in the inhibitor of nuclear factor kappa B (IκB), an inhibitor of NF-κB kinase (IKK), proteins are triggered by reactive oxygen species (ROS) (IKK). This modification leads to the degradation of IκB, leading to the release of NF-κB to translocate to the nucleus and induce the expression of several genes that express inflammatory proteins, either alone or in combination with other transcription factors [46, 47]. Therefore, inhibiting ROS-mediated signaling pathways would be one of the best treatment strategies for managing inflammatory disorders. It has been reported that kaempferol produces anti-inflammatory action by inhibiting the NF-κB signaling pathway [48]. Myricetin is another auspicious polyphenol that

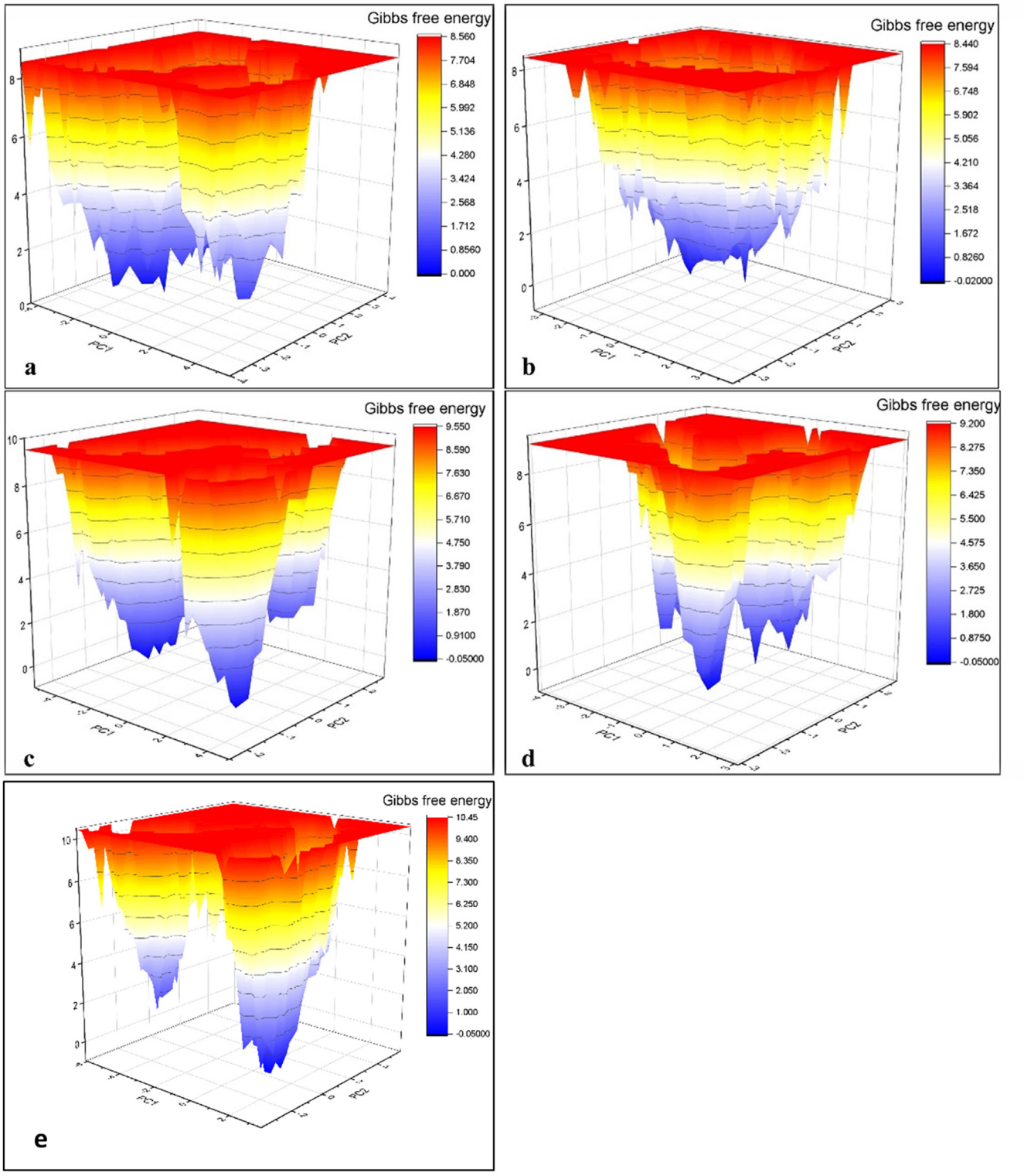

Figure 8. Gibbs free energy landscape of apo-Cox-2 and ligand bound Cox-2. (a) apo-Cox-2, (b) Cox-2 bound with celecoxib, (c) Cox-2 bound with kaempferol, (d) Cox-2 bound with caffeic acid, (e) Cox-2 bound with (-) epicatechin.

produces anti-inflammatory action through many mechanisms. It can suppress the production of various pro-inflammatory mediators, including NO, iNOS, TNF-α, IL-6, PGE2, and IL-12. It also produces anti-inflammatory action by inhibiting the NF-κB signaling pathway, activating STAT1 and Nrf2 pathways [49]. Caffeic acid can inhibit the PKC signaling pathway and suppress Fyn kinase activity and PGE2 production, down-regulating COX2 expression by inhibiting AP-1 expression and NF-κB expression activity [50]. Moreover, caffeic acid enhances the antioxidant defense system by modulating the Nrf2-keap-1 signaling pathway. A study reported that epicatechin induces anti-inflammatory effects by reducing the production of inflammatory mediators, including TNF-α, NO, IL-1β, PGE2, and IL-6, as well as the inactivation of MAPKs (JNK, ERK, and p38) NF-κB, and JAK2/STAT3 signaling pathways [51]. In this study, eight phenolic compounds have been identified

from MECCS, and it is possible that MECCS produced a remarkable anti-inflammatory effect through one of the mentioned mechanisms.

In the docking investigation, compared to celecoxib, kaempferol, (-) epicatechin, and caffeic acid displayed strong affinity and a substantial number of hydrogen bonds. During dynamics simulation, the number of hydrogen bonds with Cox-2 for the kaempferol and (-) epicatechin were similar to celecoxib. The hydrogen bond number was also consistent. (-) Epicatechin showed a minor fluctuation in RMSD. RMSF and radius of gyration for all ligands, including celecoxib, were very steady. Structural conformations of kaempferol and caffeic acid bound Cox-2 are stable in principal component analysis and Gibbs free energy landscape analysis. This evidence indicates that kaempferol, caffeic acid, and (-) epicatechin might be potential Cox-2 inhibitors. MECCS demonstrated promising in-vivo analgesic and anti-inflammatory activity due to the presence of auspicious bioactive compounds.

6. Conclusion

After analyzing the findings, it can be concluded that MECCS demonstrated remarkable analgesic and anti-inflammatory activity in their respective in-vivo test models and thus highlights the pharmacological importance of *C. calycina*. In silico molecular docking and molecular dynamics also predicted the Cox-2 inhibitory property of kaempferol, caffeic acid, and (-) epicatechin. Therefore, it can be assumed that the analgesic and anti-inflammatory effects of MECCS are due to the presence of these bioactive compounds. Specific compound isolation is recommended to uncover the exact analgesic and anti-inflammatory mechanism of MECCS.

Declarations

Author contribution statement

Tanoy Mazumder; Tarek Hasan: Performed the experiments; Wrote the paper.

Khondoker Shahin Ahmed: Performed the experiments.

Hemayet Hossain; Tushar Debnath: Contributed reagents, materials, analysis tools, or data.

Esrat Jahan; Naiemur Rahman: Analyzed and interpreted the data.

Md. Sadikur Rahman Shuvo; A. F. M. Shahid Ud Daula: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

No additional information is available for this paper.

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