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Research Article

Antiallergic activity of *Amoora cucullata* Roxb bark extract and profiling of its polyphenolic compounds: *In-vivo* and *in-silico* studies

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ABSTRACT

Present study was undertaken to evaluate the anti-allergic activity of the ethanolic bark extract of *Amoora cucullata* (EBAC) in mice followed by molecular docking analysis and also to quantify the major bioactive polyphenolic constituents by HPLC analysis. Anti-allergic activity was evaluated using Toluene-2,4-diisocyanate (TDI)-sensitized allergy model mice by determining nasal allergy-like symptoms as well as counting the total and differential leukocytes profile of blood and Broncho Alveolar Lavage (BAL) fluid. *In silico* molecular docking study was performed using Schrödinger Maestro 10.1 on eight isolated compounds against Histamine Methyltransferase receptor in order to justify the anti-allergic activity of the extract. Acute toxicity test was performed to determine the safety of the extract. HPLC analysis exhibited the presence of 3,4-dihydroxy benzoic acid, catechol, (-) epicatechin, caffeic acid, syringic acid, rutin hydrate and trans-cinnamic acid. In anti-allergic activity test, oral administration of the EBAC improved nasal-allergic symptoms of TDI-induced allergic rhinitis in experimental mice. There was also a conspicuous decrease in the number of leukocytes, neutrophils, lymphocytes, eosinophils, monocytes and basophils in both blood and Broncho Alveolar Lavage (BAL) fluid of the mice treated with EBAC as compared to TDI-sensitized mice. Among the identified compounds from this plant, the docking score of Chrysin, Apigenin, Kaempferol-3-O- beta-D-glucopyranoside, and Stigmasterol against Histamine Methyltransferase receptor were comparable with that of standard drug Cetirizine (-9.519 kcal/mol). The EBAC did not exhibit any mortality in mice even at 3 gm/kg dose. The results of this study confirm the anti-allergic activity of the plant in both *in vivo* and *in silico* investigations.

Keywords:
Amoora, Allergy, HPLC analysis, *In-silico*

1. INTRODUCTION

Amoora cucullata (Family: Meliaceae), a moderate sized mangrove tree species mostly grown in low salinity regions. It is mainly distributed in the coastal regions of Southeast Asia and the Indian Ocean especially in the mangrove regions of Bangladesh, India, Pakistan, Myanmar, Malaysia, Nepal, Thailand, Vietnam, as well as in Solomon Islands and Papua New Guinea[1]. In Bangladesh, it is found in Sundarbans east and locally known as 'Amur', 'Natmi', 'Latmi', bekak, garoti and commonly known as Amoora all over the world[2]. It possesses small white inflorescence producing fruit containing 1 to 3 seeds that are red in color. In traditional medicine, leaves are being used to reduce inflammation. In addition, juice obtained from plant has been reported to possess anti-bacterial property and used extensively for the treatment of dysentery, skin diseases and in cardiac diseases[3]. In Thailand, this plant is popular for the treatment of bone marrow, diarrhea, and inflammation[4].

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The Plant is reported to have antioxidant, antibacterial, antifungal, cytotoxic, CNS depressant, anti-inflammatory, antinociceptive and diuretic properties[5]. Moreover, scientific investigations claimed that various parts of the plant contained multifarious secondary metabolites including Alkaloids, e.g. cucullamide, dasyclamide, hydroxytigloyl-1, 4-butanediamidecyclofoveoglin[6]; Flavonoids, e.g. chrysin, apigenin; Labdane diterpenoids, e.g. *β*-hydroxylabda-8, 13,14-triene; 2*α*-hydroxylabda-8,14-triene; *ent*-13-epi-manool; *ent*-2*β*-hydroxymanool; 2*β*, 15-dihydroxy-*ent*-labda-8,13*E*-diene; Kaurane diterpenoids, e.g. kaur-15-en-17-ol[6]; Triterpenoids, e.g. betulinic acid, friedelin[7], Cycloartane triterpenopids, e.g. (24S)-21, 24,25,28-tetrahydroxycycloartane-3-one[6]; Rocaglamide derivatives, e.g. 1-O-formylrocagloic acid, 3'-hydroxy rocagloic acid, rocaglaol, rocagloic acid, 3'-hydroxymethylrocaglate, 1-O-formylmethyl rocaglate, methylrocaglate; Glycosides, e.g. kaempferol-3-O-β-D-glucopyranoside[8]; Steroids, e.g. stigmasterol, β sitosterol; Phenolic acid, e.g. caffeic acid[7].

The plant has been used to relieve skin diseases traditionally and reported to possess some potential phytoconstituents but there is no report demonstrating the anti-allergic activity of its bark. In continuation of our phytochemical screening for anti-allergic activity, here we report the anti-allergic activity of EBAC extract in both *in vivo* and *in silico* models.

2. MATERIAL AND METHODS

2.1. Collection of plant material

The barks of *Amoora cucullata*, were collected from Sundarban East, Khulna district, Bangladesh. Any type of adulteration was strictly avoided during collection. Botanical identification was performed by the experts of Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (Accession no: DACB-43148) has been kept for further reference.

2.2. Drying and grinding

The barks were cut into small pieces with sharp knife and subjected to shade drying to remove moisture. The plant materials were grinded into coarse powder using a hammer mill mechanical grinder. The powder was kept in an air tight container to avoid any possible fungal attack and then stored in a dark, cool and dry place until the extraction was started[9].

2.3. Extraction

Coarse powdered plant material was subjected to cold extraction with 95% ethanol for 7 days with regular shaking and stirring. After maceration, filtration was undertaken to remove unwanted plant material and to get clear solution. The collected filtrate was concentrated by rotary evaporator at 40°C to get crude extract[10].

2.4. Experimental animals

Young Swiss-Albino mice of both sexes (age 5-6 weeks, weight 18-25 g) were used in the present *in vivo* pharmacological investigations. They were purchased from the Animal Resources Department of Jahangir Nagar University, Bangladesh. The animals were housed under standard laboratory conditions (room temperature of 25±1°C, and relative humidity of 56-60% with 12-h day-night cycle) and had access to standard food and water *ad libitum*. They were allowed to acclimatize to the environment for 7 days prior to pharmacological experiment. All *in vivo* experiments were carried out following the guidelines of Animal Ethics Committee of Khulna University, Bangladesh.

2.5. Chemicals and drugs

Toluene-2, 4-diisocyanate (TDI) was obtained from Wako Chemical, Tokyo, Japan. Tween-80 was obtained from Loba Chemie Pvt Ltd, India. Cetirizine was obtained from Square Pharmaceuticals Ltd., Bangladesh. Gallic acid, 3,4-Dihydroxy benzoic acid, Catechin hydrate, Catechol, (-) Epicatechin, Caffeic acid, Vanillic acid, Syringic acid, Rutin hydrate, *p*-Coumaric acid, *trans*-Ferulic acid, Rosmarinic acid, Myricetin, Quercetin, trans-Cinnamic acid and Kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, acetic acid, and ethanol were of HPLC grade and obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.6. Preliminary phytochemical screening

Standard qualitative group tests were conducted to identify major groups of secondary bioactive metabolites present in crude ethanolic extract of the plant following standard testing methods[11].

2.7. Polyphenolic compounds determination by HPLC-DAD

HPLC analysis was performed on a Shimadzu (LC-20A, Japan) equipped with a binary solvent delivery pump (SIL-20A HT), an auto sampler (SIL-20A HT), column oven (CTO-20A) and a photodiode array detector (SPD-M20A) and controlled by the LC solution software (Lab Solution). Separation was performed using Luna C_{18} (5 µm) Phenomenex column (4.6×250 mm) at 33°C.

The phenolic composition of the EBAC extract was determined by HPLC following the method described by Sarunya and Sukon with little modifications[12]. The mobile phase was composed of A (1% acetic acid in acetonitrile)

and B (1% acetic acid in water) with gradient elution: 0.01-20 min (5-25% A), 21-30 min (25-40% A), 31-35 min (40-60% A), 36-40 min (60-30% A), 41-45 min (30-5% A) and 46-50 min (5% A) was used in this study. The sample injection volume was 20 µl and the flow rate was set at 0.5 ml/min. The UV detector was set at 270 nm and applied for validation of method and analysis. Stock solution of each phenolic compound was prepared in methanol containing gallic acid, 3,4-dihydroxy benzoic acid, catechin hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid, and kaempferol. The concentration of the stock solution ranged from 4.0 to 5.0 µg/ml. The appropriate volumes of each stock solution were mixed together and then diluted serially to prepare the working standard solutions. All solutions were stored under refrigeration.

A solution of EBAC was prepared in methanol having the concentration of 5 mg/ml. Prior to HPLC analysis, all solutions (mixed standards, EBAC solution and spiked solutions) were filtered through a 0.45 µm Nylon 6,6 membrane filter (India) and degassed under vacuum.

2.8. Acute Toxicity Test

Acute toxicity test was performed following the Organization for Economic Co-operation and Development (OECD) guidelines-425 with slight modifications[13]. In brief, young and healthy Swiss albino mice were divided into six groups where each group consisting of six animals. Control group received normal saline (2 ml/kg body weight) while other groups were administered 100, 200, 500, 1,000, 2,000, and 3,000 mg/kg of test extract orally. After administration, all animals were observed for 24 hours to notice any kind of behavioral changes or toxicity. Neither any sign of toxicity nor mortality was observed within 24 hours. The animals were then kept under close observation for next two weeks.

2.9. Anti-allergic activity

2.9.1. Experimental design

Fifty experimental animals were randomly selected and divided into five groups denoted as group-I, group-II, group-III, group-IV and group-V consisting of ten mice in each group. Group-I (control) received ethyl acetate (10 µl) bilaterally in the nasal vestibules and 2% tween 80 water orally. Group-II served as TDI control and was given TDI (10µl of 5% TDI solution in ethyl acetate) bilaterally in the nasal vestibules and 2% tween-80 water equivalent to the vehicle given with the extract. Group-III served as standard group and was given TDI (10 µl of 5% TDI solution in ethyl acetate) bilaterally in the nasal vestibules and antihistamine (cetirizine) 20 mg/kg body weight orally. Group-IV and group-V received EBAC orally (300 mg/kg and 500 mg/kg body weight respectively) and TDI (10 µl of 5% TDI solution in ethyl acetate) bilaterally in the nasal vestibules (Figure 1).

2.9.2. TDI sensitization and provocation

This test was carried out as per the procedure previously described by Dev et al. 2009[14]. In brief, Mice were sensitized with 10 µl of 5% TDI in ethyl acetate. The solution was applied bilaterally on the nasal vestibule of

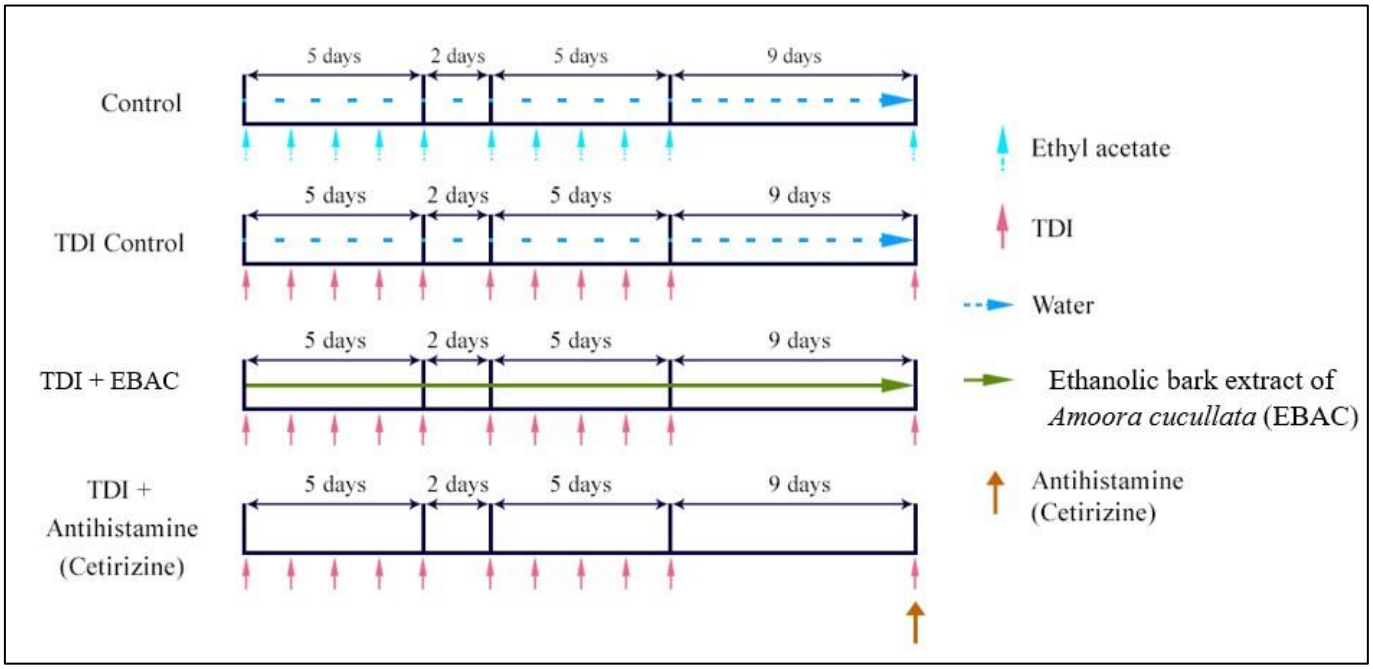

Figure 1. Experimental protocol for anti-allergic evaluation.

Table 1. Criteria for grading the severity of TDI-induced nasal responses in mice.

	Score			
Nasal response	0	1	2	3
Watery rhinorria	(−)	At the nostril	Between 1 and 3	Drops of discharges
Swelling and redness	(−)	Slightly swollen	Between 1 and 3	Strong swelling with redness

each mouse once a day for 5 consecutive days. After a 2-day interval, the sensitization procedure was repeated. Nine days after the second sensitization, 10 µl of 5% TDI solution was again applied to the nasal vestibule in order to provoke nasal allergy-like symptoms (sneezing, scratching, watery-rhinorrhea, swelling and redness). Ethyl acetate (10 µl) was applied to the mice nostril of control group during sensitization and provocation using the same procedure.

2.9.3. Assessment of Allergy-like symptoms

Nasal allergy-like symptoms (sneezing, scratching, and nasal score) were measured following the method described previously[15]. In brief, just after TDI provocation, the number of sneezes and scratches were counted carefully in each mouse for 10 minutes. The extent of watery-rhinorrhea was assessed on grading scale of 0 to 3 (0 is the lowest and 3 is the highest) (Table 1).

2.9.4. Blood collection and blood cell count

After 24 hours of 5% TDI provocation, animals of each group were anesthetized by injecting sodium phenobarbital and blood sample were collected from cervical vein[16]. Heparinized tube was used to collect blood. The collected blood was diluted with 1% acetic acid at 1:10 ratio in order to lyse the red blood cells. Then total count of WBC was conducted on automated cell counter (DS-500i, 5-part automated hematology analyzer; Edan Instrument Inc., Shenzhen, China). For differential analysis, slides were prepared using Leishman stain. After drying, slides were examined under microscope (300 cells/slide were counted on a compound microscope at 400× magnification and cells were identified as neutrophils, lymphocytes, eosinophils, monocytes and basophils using standard morphological criterion.

2.9.5. Collection of Broncho Alveolar Lavage (BAL) fluid and cell count

Prior to collection of Broncho Alveolar Lavage (BAL) fluid, lungs were lavaged for 3 times with 3 ml aliquots of 0.9% sterile saline solution via cannulated tracheal tube[17]. After the collection of BAL fluid from each mouse, it was centrifuged (1,500 rpm, 10 min at 4°C). Supernatant was separated and stored at -80°C until analysis.

2.10. In silico molecular docking study for anti-allergy activity

2.10.1. Ligand Preparation

Two-dimensional chemical structures of the selected compounds were retrieved from the PubChem, a database of freely accessible chemical information (https://pubchem.ncbi.nlm.nih.gov). Then, the ligands were prepared for the molecular docking with the LigPrep tool associated in Schodinger suite- Maestro v10.1. Following that, ligands were optimized by neutralizing at the pH of 7.0± 2.02 using Epik 2.2 and the OPLS (Optimized Potentials for Liquid Simulations)-2005 force field.

2.10.2. Protein Preparation

Three-dimensional X-ray crystal structure of Histamine Methyltransferase (PDB ID: 2AOT) was obtained from the protein data bank (PDB)[18]. After that, protein structures were prepared and refined using the protein preparation tool of SchrÖdinger suit- Maestro v10.1. Water molecules and all other undesirable residues were removed after preprocessing and then further subjected to hydrogen bond optimization and energy minimization. Charges and bond orders were assigned, hydrogens were added to the heavy atoms, selenomethionines were converted into methionines, and all waters were deleted. Using force field OPLS_2005, minimization was carried out, setting maximum heavy atom RMSD (Root-Mean-Square-Deviation) to 0.30 Å.

2.10.3. Receptor Grid Generation & Glide Ligand Docking

Receptor grids were calculated for prepared proteins such that various ligand poses bind within the predicted active site during docking. In Glide, grids were generated, keeping the default parameters of van der Waals scaling factor 1.00 and charge cutoff 0.25 subjected to OPLS 2005 force field. A cubic box of specific dimensions centered on the centroid of the active site residues (Reference ligand active site) was generated for the receptor. The bounding box was set to 14 Å×14 Å×14 Å for docking experiments.

Molecular docking study was made to reveal the possible mechanism of action of the selected compounds behind the anti-allergic activity. Standard precision (SP) flexible ligand docking experiments were performed by Glide standard precision docking using the Glide docking tool, which was embedded in Schrödinger suite-Maestro

Table 2. Phytochemical screening of EBAC.

Phytochemical group	Reagent	Results
Reducing sugar	Benedict's reagent	-
Alkaloids	Dragendroff's reagent	+
	Mayer's reagent	+
	Wagner reagent	+
Tannin	Ferric Chloride solution	+
	Lead Acetate Test	+
Flavonoids	$FeCl_3$	+
	Lead Acetate	+
Glycosides	Keller-Kiliani Test	+
	Molisch's Test	+
	Conc. H_2SO_4	+
Saponin	Froth Test	+
Gum	Molisch's reagent	+
Steroids	Salkowski's Test	+

(+) indicates Presence; (-) indicates Absence

Table 3. Polyphenolic compounds in EBAC identified by HPLC analysis.

Polyphenolic Compounds	Content (mg/100g extract)
3,4-Dihydroxy benzoic acid	17.99
Catechol	124.59
(-) Epicatechin	68.42
Caffeic acid	43.53
Syringic acid	4.70
Rutin hydrate	15.36
trans-Cinnamic acid	5.07

v 10.1. This tool applies penalties to non-cis/trans amide bonds. Van der Waals scaling factor and partial charge cutoff were selected to be 0.80 and 0.15, respectively, for ligand atoms. Final scoring was performed on energy-minimized poses and displayed as Glide score. The best-docked pose with the lowest Glide score value was recorded for each ligand.

2.11. Statistical analysis

Experimental values are expressed as mean±SEM (Standard error of mean). Statistical analysis was conducted using one-way and two-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Results were considered statistically significant at a value of $P<0.05$ in comparison to control. Prism 8.0.2 software (GraphPad software Inc., San Diego, CA, USA) was used to perform all statistical analysis.

3. RESULTS

3.1. Preliminary phytochemical screening

In phytochemical screening, EBAC exhibited the presence of alkaloids, flavonoids, glycosides, tannins, saponins, gums and steroids (Table 2).

3.2. Polyphenolic compounds determination by HPLC-DAD

HPLC analysis of EBAC exhibited the presence of 3,4-dihydroxy benzoic acid, catechol, (-) epicatechin, caffeic acid, syringic acid, rutin hydrate, trans-cinnamic acid with the concentrations of 17.99, 124.59, 68.42, 43.53, 4.70, 15.36 and 5.07 mg/100 g of EBAC, respectively (Table 3). Gallic acid, catechin hydrate, vanillic acid, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin and kaempferol were absent or present in a trace amount beyond the detection limit (Figure 2 and Figure 3).

3.3. Acute toxicity study

The extract did not produce any mortality or any kind of toxicity throughout the study period of 14 days even at 3,000 mg/kg body weight. This result revealed the non-toxic nature of the extract.

3.4. Anti-allergic activity study

3.4.1. Effect on TDI-induced nasal allergy like symptoms

In TDI-sensitized mice (TDI-control group), the total number of sneezes (Figure 4a), scratches (Figure 4b) and the nasal score (Figure 4c) were 37.16±1.35, 266.16±15.9, 3±0 respectively. While the number of sneezes, scratching and nasal score for standard cetirizine were 15.67±1.58, 113.17±10.36, 0.67±0.21 respectively; for 300 mg/kg dose of EBAC those values were 32.33±0.95, 186±23.74, 1.67± 0.21 respectively whereas for 500 mg/kg dose of EBAC those values were 17.5±1.48, 145.83±12.84, 0.83±0.17

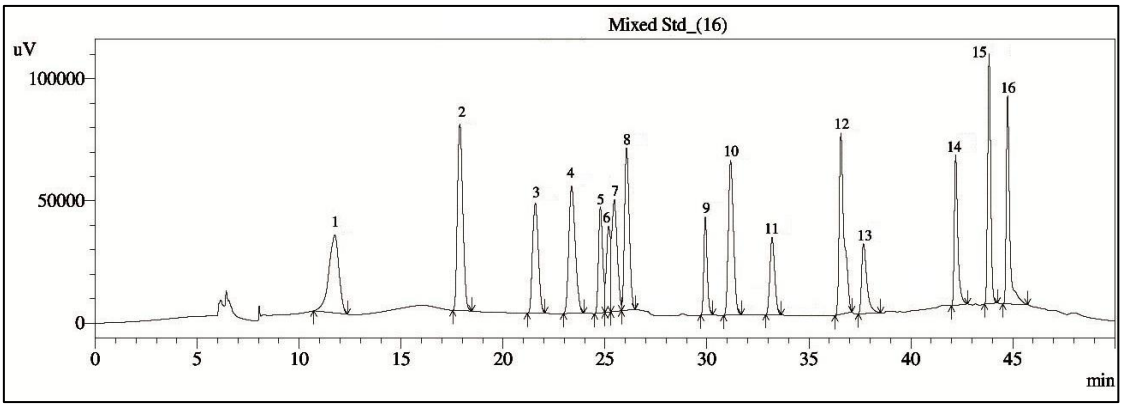

Figure 2. HPLC Chromatogram of a mixture standard of polyphenolic compounds. Here peaks indicate, 1: Gallic acid, 2: 3,4 dihyrdoxy benzoic acid, 3: Catechin hydrate, 4: Catechol, 5: (-) Epicatechin, 6: Caffeic acid, 7: Vanillic acid, 8: Syringic acid, 9: Rutin hydrate, 10: p-Coumaric acid, 11: Trans-Ferulic acid, 12: Rosmarinic acid, 13: Myricetin, 14: Quercetin, 15: Trans-Cinnamic acid, 16: Kaempferol.

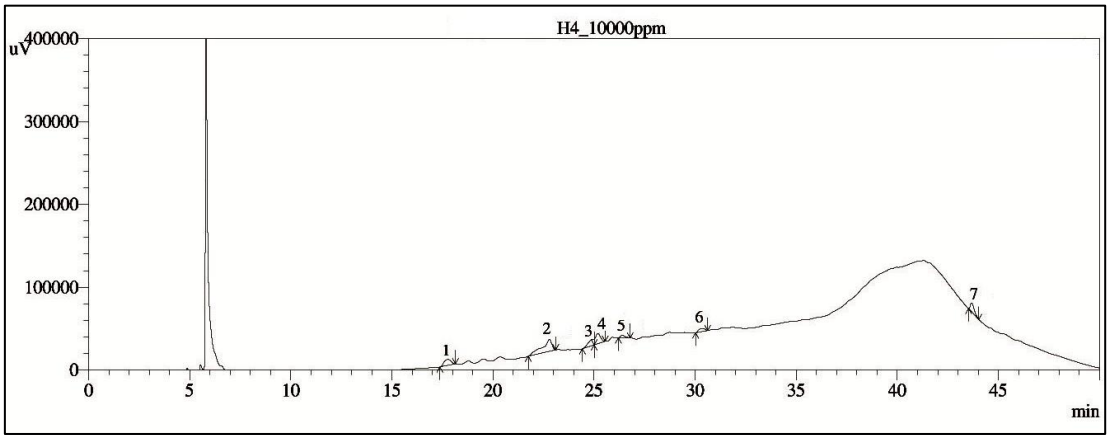

Figure 3. HPLC Chromatogram of EBAC. Here peaks indicate, 1: 3,4-Dihydroxy benzoic acid, 2: Catechol, 3: (-) Epicatechin, 4: Caffeic acid, 5: Syringic acid, 6: Rutin hydrate, 7: trans-Cinnamic acid.

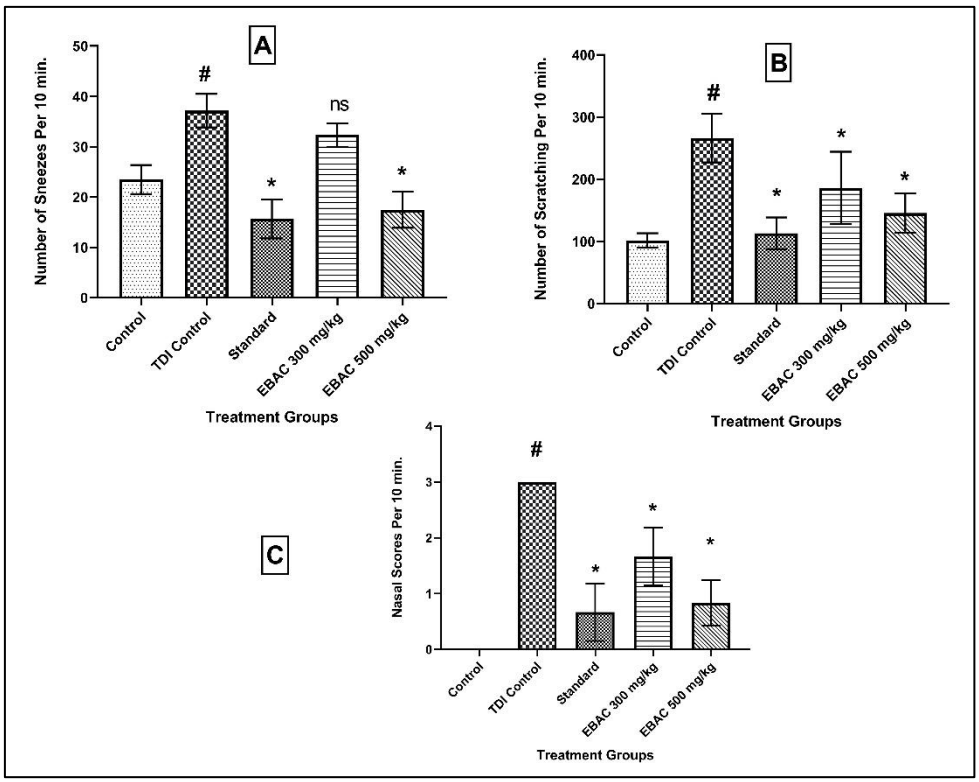

Figure 4. Suppressive effect of EBAC on Sneezing (a), Scratching (b), and Nasal Score (c). Values are expressed as Mean±SE, n=10, $^{\#}p<0.05$ vs non-sensitized control (ethyl acetate); $^{*}p<0.05$ vs TDI Control (TDI sensitized control); ns-indicates non-significant.

respectively. Oral administration of EBAC for 3 weeks significantly decreased the sneezes, scratches and nasal scores and the efficacy of the extract was comparable with the standard antihistamine (cetirizine, 20 mg/kg) at the dose of 500 mg/kg.

3.4.2. Effect on total and differential blood cell count

In blood samples of TDI-control mice, the total number of circulating leukocytes, neutrophils, lymphocytes, eosinophils, monocytes and basophils were markedly increased as compared to that of control group. There was a noticeable decrease in the number of leukocytes, neutrophils, lymphocytes, eosinophils, monocytes and basophils in the blood of the mice treated with EBAC as well as cetirizine in comparison with the levels of in TDI-sensitized mice. The efficacy of the extract was comparable with the standard cetirizine (Figure 5).

3.4.3. Infiltration of WBC in BAL fluid

The differential analysis of bronchoalveolar lavage (BAL) fluid revealed that oral administration of EBAC significantly decreased the total count of WBC and the number of neutrophils, lymphocytes, eosinophils, monocytes and basophils in the broncho alveolar fluid of the mice in a dose dependent manner when compared with positive control group. The efficacy of extract at a dose of 500 mg/kg was comparable with the standard drug cetirizine (Figure 6).

3.4. In silico molecular docking study for anti-allergy activity

In this studies, eight selected potential compounds isolated from *Amoora cucullata*, were docked with Histamine Methyltransferase (PDB ID: 2AOT) receptor for

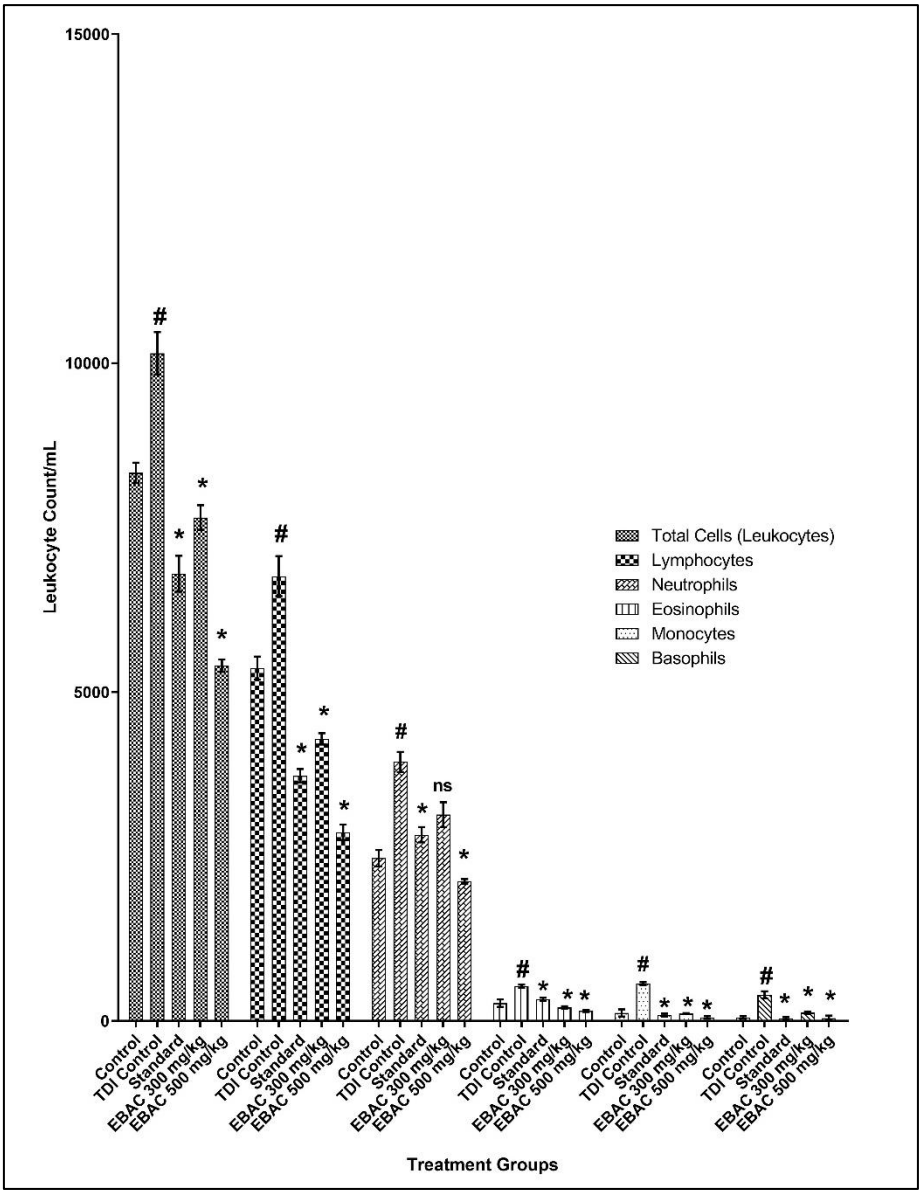

Figure 5. Suppressive effect of EBAC on total blood cells (leukocytes) and differential cells count in blood of TDI-sensitized mice. Values are expressed as Mean±SE, n=10, #p<0.05 vs Control; *p<0.05 vs TDI Control; ns-indicates non-significant.

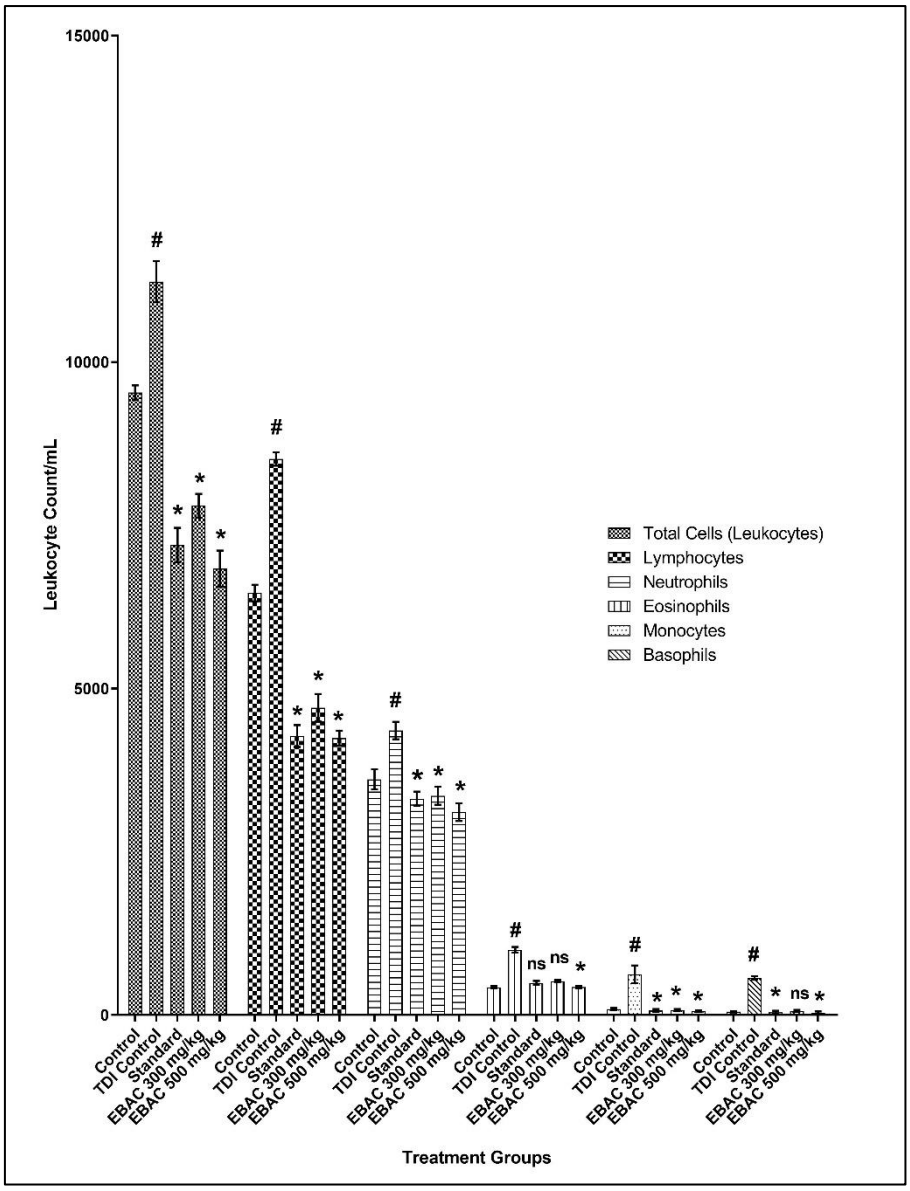

Figure 6. Suppressive effect of EBAC on total blood cells (leukocytes) and differential cells count in bronchoalveolar lavage fluid of TDI-sensitized mice. Values are expressed as Mean±SE, n=10, #p<0.05 vs Control; *p<0.05 vs TDI Control; ns-indicates non-significant.

anti-allergy activity. From the result, it was observed that among the eight compounds, stigmasterol has the best binding affinity against the receptor with the docking score of -9.398 kcal/mol followed by kaempferol-3-O- beta-D-glucopyranoside (-8.968 kcal/mol), apigenin (-8.728 kcal/mol), chrysin (-8.569 kcal/mol) and caffeic acid (-6.194 kcal/mol). Whereas docking score for the standard drug, cetirizine against the receptor was -9.519 kcal/mol. Rest three compounds namely β sitosterol, friedelin and betulinic acid did not dock with this enzyme. The results of the docking analysis for anti-allergic activity had been presented in Table 4 and docking figures in Figure 7, Figure 8, and Figure 9.

4. DISCUSSION

Allergy involves hyperactive or hypersensitive immune response to foreign objects like chemicals, certain foods, pollen, fur, dust or other allergens, causing sneezing, itching, rashes, runny nose, swelling or asthma. In traditional medicine, herbal drugs from various medicinal plants have been used widely to prevent or ameliorate the allergic symptoms[19]. People of rural areas are using these herbal drugs traditionally over the years due to the availability of the medicinal plants as well as high expenses and side effects of allopathic drugs. Several medicinal plants are popular as healing agents for allergic disorders without any scientific evaluation[15]. Since traditional medicine could be a reliable source of drug discovery and development, researchers are prone to explore these valuable natural resources in order to find out more improved therapy for allergic conditions.

In order to elucidate the anti-allergic effect of EBAC, Toluene-2, 4-diisocyanate (TDI), widely used industrial chemical associated with allergic diseases[20], was used to induce allergic conditions in mice. Intranasal application

Table 4. Docking score of selected compounds against Histamine Methyltransferase for antiallergic activity.

Compound Name	Pubchem ID	Docking Score (kcal/mol)
Chrysin	5281607	-8.569
Apigenin	5280443	-8.728
Kaempferol-3-O- beta-D-glucopyranoside	21606527	-8.968
Stigmasterol	5280794	-9.398
β-sitosterol	222284	-
Caffeic acid	689043	-6.194
Friedelin	91472	-
Betulinic acid	64971	-
Standard Drug		
Cetirizine	2678	-9.519

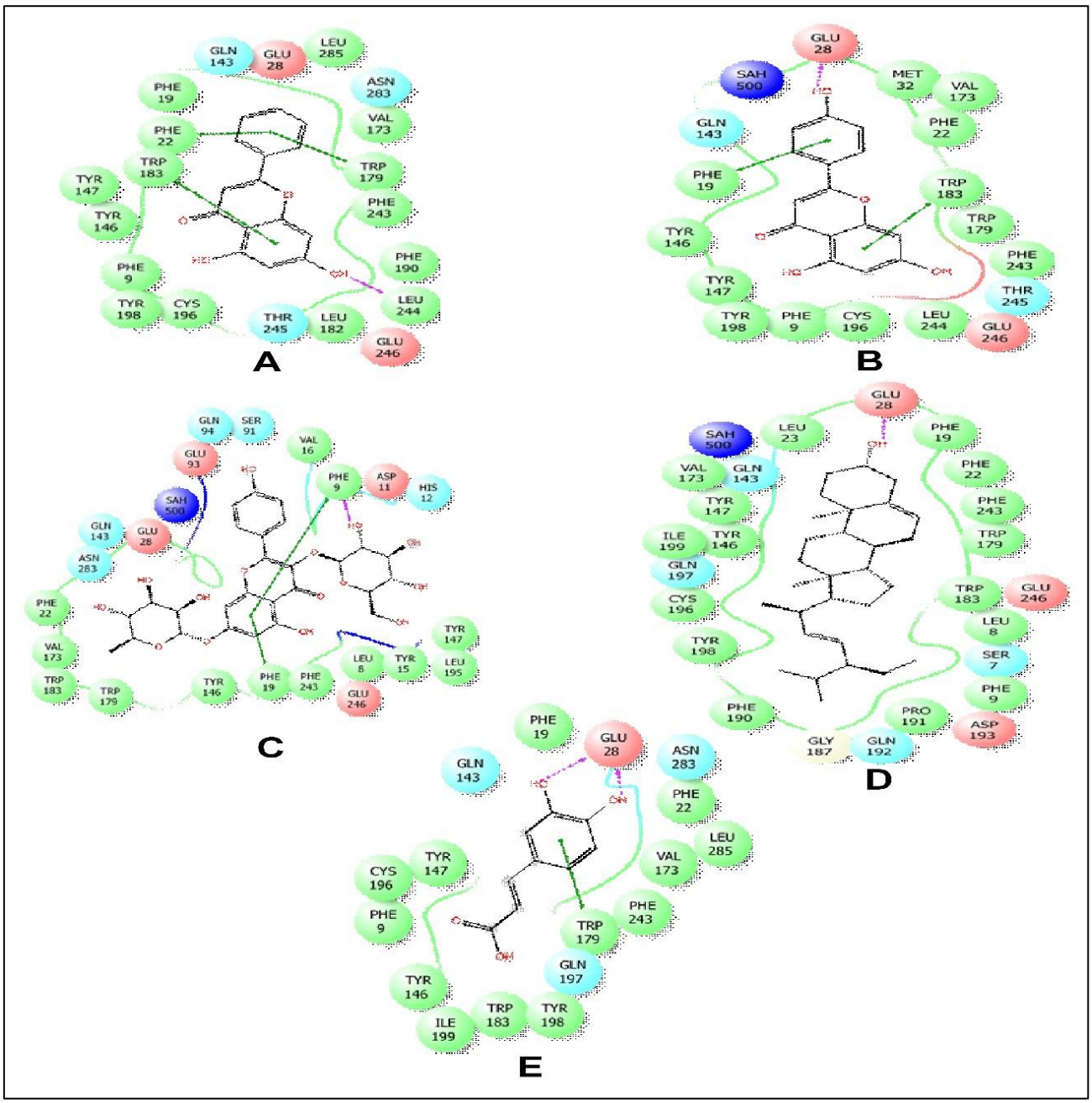

Figure 7. 2D interactions of chrysin (A), apigenin (B), kaempferol-3-O- beta-D-glucopyranoside (C), stigmasterol (D) and caffeic acid (E) with Histamine Methyltransferase (PDB ID: 2AOT). Colors indicate the residue (or species) type: Red-acidic, Green-hydrophobic, Purple-basic, Blue-polar, Light gray-other 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.

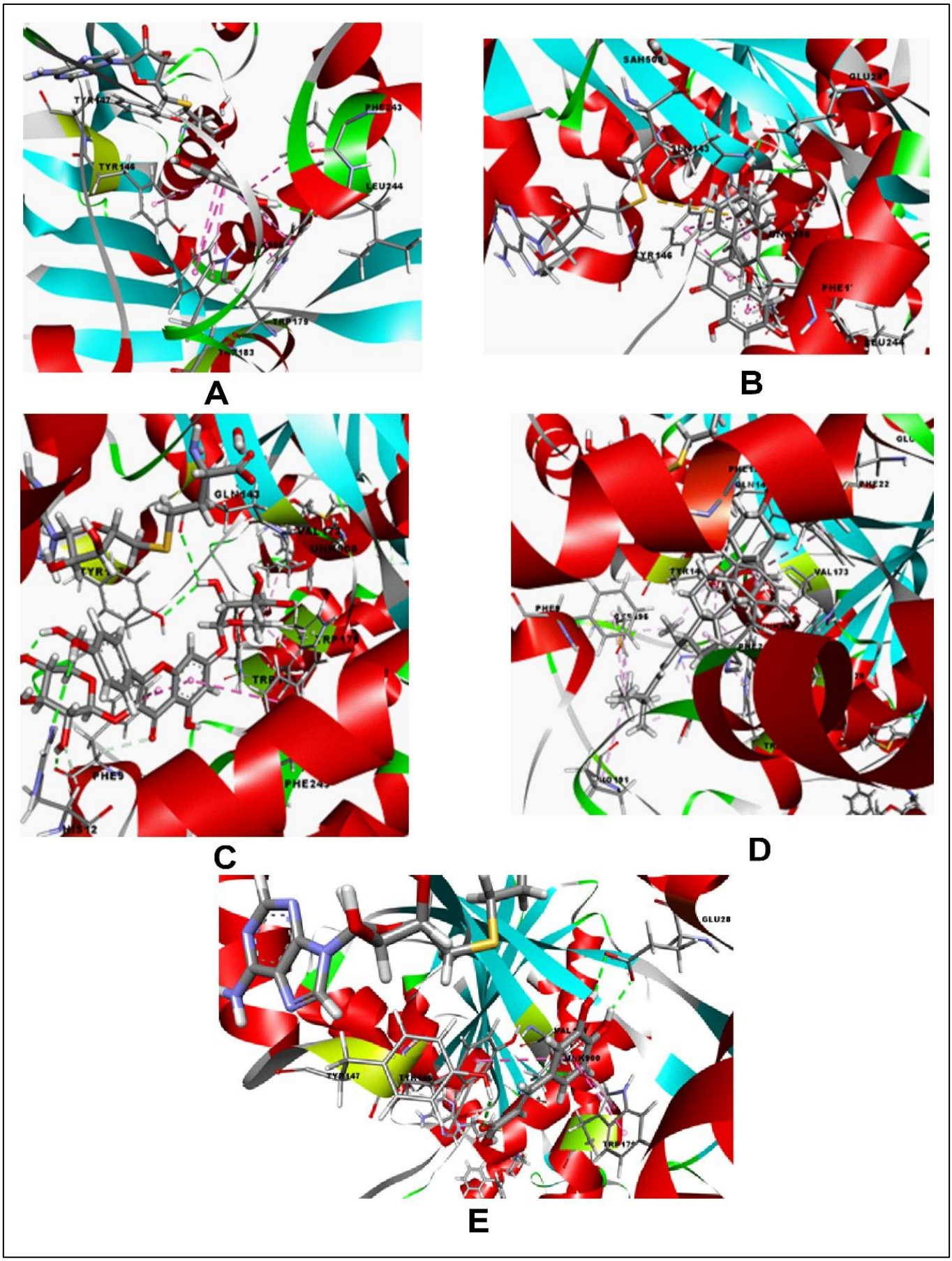

Figure 8. 3D interactions of chrysin (A), apigenin (B), kaempferol-3-O- beta-D-glucopyranoside (C), stigmasterol (D) & caffeic acid (E) with Histamine Methyltransferase (PDB ID: 2AOT).

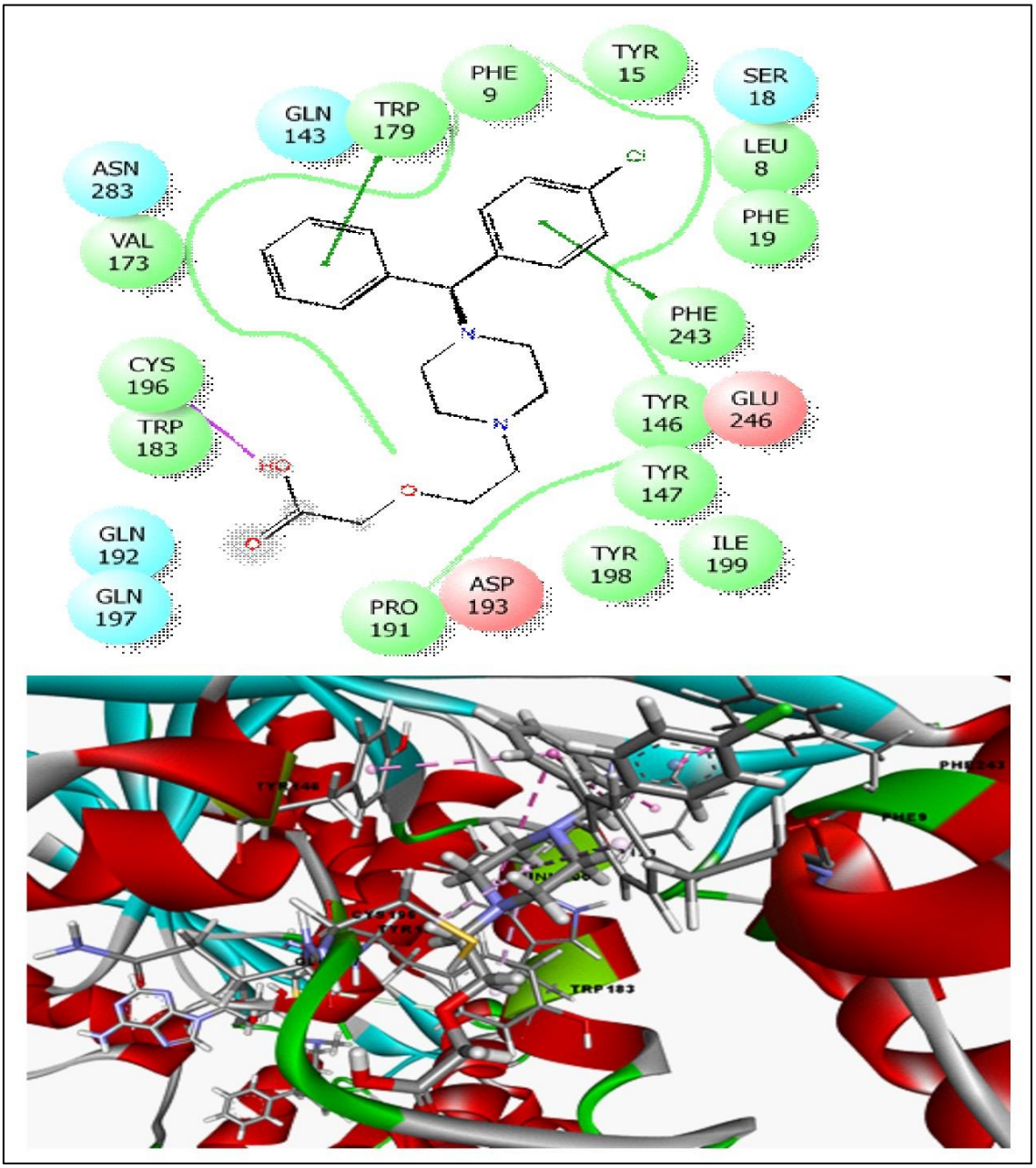

Figure 9. 2D & 3D interactions of Cetirizine (standard drug) with Histamine Methyltransferase (PDB ID: 2AOT). In 2D picture Colors indicate the residue (or species) type: Red-acidic, Green-hydrophobic, Purple-basic, Blue-polar, Light gray-other and Darker gray-metal atoms. Interactions with the protein 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.

of TDI causes neuropeptide-mediated release of histamine from the mast cells in the nasal mucosa and induces the allergy like symptoms e.g., redness, swelling, nasal blockage, sneezing, scratching (rubbing) and watery rhinorrhea. In addition, it also displays some characteristic features of allergic rhinitis including differential cells infiltration in the tissues of lungs and airways, elevated levels of Th_2 cytokines like IL-4, IL-6 etc.[21], histamine H_1 receptor (H_1R) mRNA and protein[22]. Moreover, histamine is considered to be one of key mediators responsible for the initiation and development of allergic reactions[23]. Researchers in several studies mentioned that activation of H1R by histamine is one of the important factors responsible for allergy-like symptoms (sneezing, nasal itching, watery rhinorrhea)[24]. In the present investigation, EBAC exhibited symptomatic relief of allergic rhinitis in mice by significantly suppressing sneezing, scratching and nasal score as shown in Figure 4a, Figure 4b, and Figure 4c. Based on these findings, we can assume that the extract might possess some suppressive role on the above-mentioned parameters which might improve the allergic symptoms and produce symptomatic relief of allergic rhinitis in TDI-sensitized mice.

Eosinophils are multifunctional cells, considered as

one of the key components of the immune system and important sources of various regulatory and inflammatory cytokines (IL-2, IL-3, IL-4, IL-5, IL-6 etc.), chemokines (CCL3, CCL5), tumor necrosis factors (TNF-α), interferon-gamma (IFN-γ)[25]. Moreover, eosinophil secrets four major cationic proteins: eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and major basic protein (MBP), all of which can cause damage to the tissues[26]. In allergic disorders, considerable amount of eosinophil production occurs by the bone marrow leading to eosinophilia (accumulation of eosinophil in blood)[27]. During allergic inflammation, IL-5 is the major cytokine responsible for the accumulation of eosinophils in the blood and plays a key role for the migration of the eosinophils from bone marrow to the blood[28]. In fact, increased number of eosinophils and leukocytes can be found in the blood of allergic and asthmatic patients which is considered to be a hallmark for allergic disorders.

Neutrophils are short-lived and terminally differentiated cells are found in elevated levels in peripheral blood, nasal lavage fluid (NAL), nasal biopsies and broncho alveolar lavage (BAL) fluid of the patients with allergic rhinitis[29]. Researchers reported that activated and differentiated neutrophil subsets accumulate in the site of allergic provocation and greater number of neutrophils are found both locally and systemically in symptomatic allergic rhinitis; these findings are consistent with our present investigation. Apparently, in our study, we have found elevated levels of total leukocytes, eosinophils, lymphocytes, neutrophils, basophils and monocytes in both blood and BAL fluid of TDI-control mice (Figure 5 and Figure 6) whereas treatment with the EBAC at a dose of 300 mg/kg and 500 mg/kg and standard drug cetirizine (20 mg/kg) reduced the count of these inflammatory cells in allergic mice. Therefore, it is evident that the extract has significant suppressive effect on the cells linked to inflammation in TDI-sensitized allergy mice model. Nearly similar studies had been done by the several researchers who used TDI for inducing asthma in guinea pig and rat and the findings are consistent with our study.

Another probable mechanism of the anti-allergic activity of EBAC might be due to inhibitory activity on the release of allergen-specific immunoglobulin (IgE) or due to the protective effect of the extract on the mast cells from degranulation in sensitized mice. A similar mechanism has been proposed for anti-asthmatic properties of the extract mixture of *Glycrrhiza glabra, Adhatoda vasica, Solanum xanthocarpum;* roots of *picrorhiza kurroa*, bark of *Albizzia lebbeck*[30]. In the present study, the effect of EBAC on IgE or mast cells was not checked due to facilities limitation.

The phytochemical analysis of EBAC showed the presence of alkaloids, flavonoids, glycosides, tannins, saponins, gums and steroids (Table 2). All of them are well known to have medicinal as well as biological activities[31].

Different alkaloids like warifteine, piperine etc have been reported to possess anti-allergic activity on OVA-induced allergic rhinitis in mice[32-33]. Several flavonoids like luteolin, ayanin, apigenin, fisetin, acacetin, chrysin, hesperidin etc. have been reported beneficial effects on experimental mouse model of OVA-induced allergic asthma[34-35]. Chrysin had been isolated from *A. cucullata*[8], therefore, it might impart anti-allergic activity observed in our investigation.

A study reported catechin from the standardized extract of *Albizia lebbeck*, as the key phytomarker responsible for imparting anti-allergic activity on experimental mice[32]. Epigallocatechin-3-O-gallate and Epigallocatechin-3-O-(3-O-methyl) gallate, tannic acid, apple condensed tannins[33], ameliorate allergic conditions. Caffeoylxanthiazonoside isolated from fruits of *Xanthium strumarium*, showed significant anti-allergic activity on rats having allergic rhinitis[34]. Chong et al. reported that saponins, such as gleditsioside I, pitheduloside A, pitheduloside E, vitalboside A, isolated from the fruits *Gleditsia sinensis*, prevented the mast cell degranulation of rats possibly through elevating intracellular cAMP levels[35]. The plant is reported to contain stigmasterol (stigmast-5, 22-dien-3β-ol)[7] and in a recent study, combination of stigmasterol and dexamethasone (anti-allergic drug), showed more potent anti-allergic activity than the dexamethasone alone in TDI-induced allergic conditions in Wister rats[16]. Additionally, considerable amount of (-) epicatechin, caffeic acid, syringic acid, rutin hydrate, were identified in HPLC analysis of the EBAC extract (Table 3), which showed anti-allergic activity in previous studies[32]. Therefore, identified polyphenols and other phytochemical groups might combinedly contribute in the observed anti-allergic activity of EBAC extract.

In silico docking study not only helps to predict the active site of a protein or enzyme but also helps to understand the binding mood of the compounds with the active site[36]. According to the molecular docking study among the eight compounds, docking score of chrysin, apigenin, kaempferol-3-O- beta-D-glucopyranoside, and stigmasterol were not significantly different from the standard drug cetirizine (-9.519 kcal/mol). Moreover, all four compounds had been reported for their anti-allergic effect[16,37-39] . So, the anti-allergic activity exerted by EBAC might be the combined effect of the above mentioned compounds.

5. CONCLUSION

In the present study, *Amoora cuculata* bark extract revealed significant amelioration of nasal allergy like symptoms as well as inhibitory activity on migration of WBC both in blood and BAL fluid. Chrysin, apigenin, kaempferol-3-O- beta-D-glucopyranoside, and stigmasterol could be the possible active compounds behind its anti-allergic activity. Further advanced investigations and

bioactivity guided studies will be required to isolate the active principle(s) for the development of better medicine for allergy and asthma.

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Conflict of Interest

The authors declare no conflict of interest.

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Ethics Approval

Our study was approved by the Research Ethics Committee of Pharmacy Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh. The approval number was KU/PHARM/AEC/15/06/ 029.

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Authors Contribution

The submitted research work was conducted in collaboration among the authors. The research was designed by SD (Shrabanti Dev), AKD (Asish Kumar Das) and IM (Imran Mahmud). IM have performed the extraction, acute toxicity study, and anti-allergic activity study. RNA (Rabindra Nath Acharyya) conducted the detail literature review and assisted in the all-laboratory test. Article writing and *in-silico* molecular docking was done by AP (Arkajyoti Paul), AMR (Md. Abid Muktadir Risha), IM and RNA. AKB (Apurbo Kumar Barman) carried out the statistical analysis. Critical revision of the article was done by NNB (Nripendra Nath Biswas), SD and AKD. All authors have read and approved the final manuscript.

Abbreviations

EBAC: Ethanolic bark extract of *Amoora cucullata*
ECP: Eosinophil Cationic Protein
EDN: Eosinophil Derived Neurotoxin
EPO: Eosinophil Peroxidase
IC_{50}: Inhibitory Concentration for 50% Test Animal
IFN-γ: Interferon-gamma
IgE: Immunoglobulin-E
IL: Interleukin
LC_{50}: Lethal Concentration for 50% of Test Animal
LD_{50}: Lethal Dose for 50% of Test Animal
Min: Minutes
PBS: Phosphate-Buffered Saline
MBP: Major Basic Protein
NAL: Nasal Lavage Fluid
SEs: *Staphylococcal* Enterotoxins
SEM: Standard error for mean
SPEs: *Staphylococcus pyogens* Enterotoxins
TDI: Toluene-2,4-diisocyanate
TNF-α: Tumor Necrosis Factors-alpha

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