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Exploration of analgesic, laxative and immunomodulatory effects of leaves and twigs of *Euphorbia tirucalli* along with *in silico* analysis

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Abstract
This study was conducted on *Euphorbia tirucalli*, a herb from Euphorbiaceae family in order to assess its some pharmacological effect based on its traditional uses. Phytochemical analysis revealed the presence of various types of phytochemical groups. Total phenolic, flavonoid and tannin contents in this extract were 111.4 mg gallic acid equivalence/g, 105.94 mg quercetin equivalence/g and 67 mg gallic acid equivalence/g, respectively. In acetic acid-induced writhing method, this extract reduced the writhing reflex up to 65% at 400 mg/kg dose. This extract increased the stool weight up to 64% in 200 mg/kg dose. In the delayed type hypersensitivity assay, this extract showed immunomodulatory effect up to 17.74% by increasing footpad thickness in 400 mg/kg dose. From molecular docking analysis, we have found 4 compounds showed good binding affinities in anti-inflammatory and laxative tests. These experimental results justify its uses in traditional medicine and these will help to conduct higher researches on this herb.

Keywords: *E. tirucalli*, antioxidant, analgesic, laxative, immunomudulatory, molecular docking

Introduction
The dependence on medicinal plants in order to get from many diseases is as old as human civilization. In the very ancient times, man was very helpless to nature in case of suffering from many life-threatening diseases. With the dependence on different medicinal plants, men started to get cure and become well. From that time to till today, numerous medicinal plants from all over the world have been used to combat diseases. Although there are significant developments in synthetic chemistry and computer aided drug designing system, still medicinal plant are used most due to their better efficacy and less side effects.

The family Euphorbiaceae is large one in plant division which consists of mainly shrubs or tint trees. Many important medicinal plants belong to this family have significant therapeutic uses in different diseases. *Euphorbia tirucalli* is such an important medicinal herb from this family (Figure 1). It is indigenous to the temperate regional countries in the world like India, Srilanka, Bangladesh, Indonesia, Vietnam, Malaysia, Angola, Mozambique, South Africa, Brazil, Colombia etc. [1]. Due to having many pencil like twigs, it is also known as 'pencil tree'. Sometimes, it is also grown as hedge plant in many gardens [2]. This plant is tall up to 5 m, having erect branches. This plant generates white extrude upon cutting. This plant is evergreen as its stems are branches are always green around a year. Its small leaves are spotted, oblanceolate about 1.3-2.5 cm in length. These are found only at tips of juvenile branchlets. Its dried stems are greenish brown, cylindrical with 0.5-2 cm in diameter. Its flowers are tiny, bottle green prearranged in many groups. A thick cluster of cyathia that only increases in chaps blooms is produced by forking two to four times with heat applied for less than one millimeter. In October, *E. tirucalli* bears blooms, then in November and December, it starts to bear fruits. Its 1 cm long fruits are capsule shaped. Many important bioactive compounds have been reported from this plant. In a previous article, Mali and Panchal, 2017 reported the presence of 12-deoxyphorbol, ingenol, *β*-sitosterol, 12-deoxy-4*β*-hydroxyphorbol-13- phenyl acetate-20-acetate, 4-deoxyphorbol, cycloeuphordenol, cyclotirucanenol, tirucalicine, tri-methyl ellagic acid, taraxasterol, tirucallol, *β*-amyrin etc. from its latex and leaves [1].

Stem and barks contain hentriacontene, 4-deoxyphorbol ester, hentriacontanol, *β*-sitosterotchouc, cyclotirucanenol, cycloeupordenol, corilagin, casuarin, euphorbins, euphorcinol, euphorginol, taraxerol, stigmasterol, ellagic acid, taraxasterol, 3, 30-di-*O*-methylellagic acid, *β*-sitosterol and some other compounds [1]. This plant have many therapeutic importance in the treatment of many diseases. Milky juice of this plant is alexiteric and shows carminative properties. The latex of *E. tirucalli* is utilized as an antibacterial agent in northeastern Brazil. The Native Americans also use it in treating asthma, cough, earache, rheumatism, epithelioma, immunosuppression and sarcoma. In east Africa, this plant is used against toothache, snake bites, hemorrhoids. Roots along with coconut oil are boiled and used in stomach pain. In India, its latex are used as emetic and anti-syphilic. In Indonesia, the leaves are used to treat nose ulcers, hemorrhoids and extraction of thorns [1-3]. Depending on its several therapeutic importance in treating different diseases we decided to conduct its leaves and small twigs antioxidant, analgesic, anti-inflammatory, laxative, immunomodulatory effects along with *in silico* analysis.

Fig 1: *Euphorbia tirucalli*

Materials and Methods

Plant collection and extraction

Fresh mature leaves and small twigs of *E. tirucalli* was collected from different areas of Khulna, Bangladesh in February, 2021. During collection all types of adulterants were carefully removed. Plant samples were sliced into small pieces and shade dried for around 2 months. Dried plant samples were identified by Professor A.K. Fazlul Hoque, Forestry and Wood Technology Discipline, Khulna University. On the other hand, remaining dried plant samples were pulverized to have coarse powder. Cold extraction was carried out with 95% ethanol in which 300 gm powdered *E. tirucalli* was macerated. After 14 days, with a rotary evaporator, 7.2 gm (yield=2.4%) gummy crude extract of *E. tirucalli* was obtained and those extract was used to conduct all of the experiment mentioned in this manuscript.

Chemicals and reagents

Analytical grade reagents such as Glacial acetic acid (Merck, Germany), Folin-Ciocalteu reagent (Merck, India), Quercetin, (Sigma Aldrich, USA), gallic acid (Sigma Aldrich, USA), Na_2CO_3 (Loba, India), $NaNO_2$ (Loba, India), $AlCl_3$ (Loba, India), NaOH (Loba, India) were used to conduct the experiments. Standard drugs like diclofenac Na, bisacodyl and levamisole were purchased from Square Pharmaceuticals Limited, Opsonin Pharmaceuticals Limited, Acme Laboratories Limited, Bangladesh, respectively.

Animal

In order to carry out the pharmacological tests, male Swiss albino mice (aged 30-40 days, average body weight: 20-25 gm) were purchased from Pharmacology laboratories of Jahangirnagar University, Dhaka, Bangladesh. Those mice were shifted to Animal house of Khulna University. To get adapted, mice were kept 2 weeks and during that time, those were fed with optimum rodent foods and pure distilled water. Mice were kept 12 hour dark-12 hour light environment. All of the mentioned tests in this manuscript were carried out in hygienic condition and the ethical permission was granted from Animal Ethics Committee, Khulna University, Khulna-9208, Bangladesh [Ref: KUAEC-2023-04-02]. In every test concerned with mice, each group was consisted with 5 mice.

Experimental site

All of the experiments were performed in the Phytochemistry and Pharmacology Laboratories, Khulna University, Khulna, Bangladesh.

Phytochemical investigation

A qualitative phytochemical test was conducted according to the method described by Kundu *et al*., 2022 [4]. From these tests, we can identify about the presence or absence of different phytochemicals groups like reducing sugars, alkaloids, saponins, phenols, glycosides, terpenoids, gums, xanthoproteins etc.

Estimation of total content of different secondary metabolites

Estimation of Total Phenolic Content (TPC)

Phenols are the mostly abundant antioxidant compounds in plant kingdom. Most of the plants contain different types of polyphenolic compounds. TPC of *E. tirucalli* extract was estimated using Folin-Ciocalteu reagent according to the method described by Jahan *et al*., 2021 [5]. The standard used in this assay was gallic acid. In brief, gallic acid was prepared at different concentrations (0.02-0.15 mg/ml) and *E. tirucalli* was prepared at 1 mg/ml while methanol was used as solvent. 0.5 ml of different concentrations of gallic acid and *E. tirucalli* extract was separately mixed with 5 ml diluted (1:10) FC reagent and 4 ml of 7% Na_2CO_3 (w/v). After vortexing these mixture for 15 seconds, UV absorbance was taken at 765 nm wavelength. From gallic acid calibration curve, we determined TPC which was expressed as mg gallic acid equivalent/gm of dried extract.

Estimation of Total Flavonoid Content (TFC)

Flavonoids are another type of antioxidant compounds which are mostly show different therapeutic response. TFC of *E. tirucalli* extract was measured using aluminum chloride colorimetric method described by Golder *et al*., 2020 [6]. The standard used in this assay was quercetin. In brief, quercetin was prepared at different concentrations (0.00-1 mg/ml) and *E. tirucalli* was prepared at 1 mg/ml while methanol was used as solvent. 1 ml of these prepared solutions of quercetin and *E. tirucalli* extract were added with 0.3 ml of 5% w/v $NaNO_2$, 0.3 ml of 10% w/v $AlCl_3$ (w/v) and finally 2 ml of 1M NaOH (w/v). Finally, distilled water was added to make it 10 ml. After an interval of 15 minutes, UV absorbance was taken at 510 nm wavelength. From quercetin calibration curve, we determined TFC which was expressed as mg quercetin equivalent/gm of dried extract.

Estimation of Total Tannin Content (TTC)

Tannins are another group of antioxidant compound found in many medicinal plants. TTC of *E. tirucalli* extract was

determined using Folin-Ciocalteu reagent according to the method described by Tambe *et al*., 2014 [7]. The standard used in this assay was gallic acid. In brief, gallic acid was prepared at different concentrations (0.02-0.15 mg/ml) and *E. tirucalli* was prepared at 1 mg/ml using methanol as solvent. 0.5 ml of different concentrations of gallic acid and *E. tirucalli* extract was separately mixed with 5 ml diluted (1:10) FC reagent and 4 ml of 7% Na_2CO_3 (w/v). Finally, volume of those solution were made 10 ml by adding distilled water. After vortexing these mixture for 15 seconds at 30 minute interval, UV absorbance was taken at 725 nm wavelength. From gallic acid calibration curve, we determined TTC which was expressed as mg gallic acid equivalent/gm of dried extract.

Evaluation of acute toxicity test

According to the OECD guidelines 425 (up and down procedure), the acute toxicity of *E. tirucalli* extract was conducted. A single oral dose of 500, 1000 and 2000 was administered to different mice groups and those were regularly monitored for the next 14 days. During the observation period, any changes in their regular activities like seizure, tremor, sedation, convulsion were carefully monitored.

Evaluation of peripheral analgesic property

By following acetic acid-induced writhing method described by Golder *et al*., 2020 we had evaluated peripheral analgesic effect of *E. tirucalli* extract [6]. *E. tirucalli* extract was orally given in two mice groups at 200 and 400 mg/kg. Diclofenac Na at 25 mg/kg was orally administered in positive control group and mice of negative control group was administered with 1% v/v tween 80 solution. After an interval of 30 minutes, 0.7% v/v acetic acid was intraperitoneally administered to each mice at 0.01x dose (x=body weight of mice in gm). Fifteen minutes later, no. of writhing (abdomen constriction, hind leg extension) was counted for 5 minutes. Inhibition of writhing no. was considered as analgesic effect.

Evaluation of laxative effect

Laxatives are first choice of drug in treating constipation. The laxative effect of *E. tirucalli* extract was determined by the method described by Saha *et al*., 2021 [8]. Mice of control group was administered with 1% v/v tween 80 solution. *E. tirucalli* extract at 200 and 400 mg/kg and bisacodyl at 10 mg/kg (as standard) was orally administered. Before conducting the experiment, all mice were kept fasting for 10 hours (only water was given). Each mice group was kept in separate metabolic cages for the next 16 hours. Excreted stools were collected and their weight was calculated. As the weight of stool was found in a cumulative data from each group, so we conducted this test 3 times to get accurate result.

Evaluation of delayed type hypersensitivity

This test was done by using sheep red blood cells (SRBC) induced sensation in mice model described by Nfambi *et al*., 2015 [9]. Mice were grouped in 5 groups. Group I was served as control group and were subcutaneously treated with 1% tween 80 at 10 ml/kg dose. Mice in the test groups (Group II–V) were treated with dexamethasone (an immunosuppressant drug) at single dose of 1.25 mg/kg dose on day 0 of the study by subcutaneous injection. Mice of negative control group (group-II) were only treated with normal saline solution. Levamisole at the dose of 50 mg/kg body weight was subcutaneously administered in mice of positive control group (group-III). Moreover, *E. tirucalli* extract was given at 200 and 400 mg/kg doses to test groups IV-V. All the treatments were continued at a single dose for a period of 7 days. On the 8th day, sheep blood was collected using ethyldiamine tetra acetic acid as anticoagulant from a local slaughterhouse and those were centrifuged later at 2000 rpm for 10 minutes. After 4 time washing with 0.9% NaCl solution, the washed SRBS were suspended in buffered saline at 20% concentration. On day 8th of the study, each mouse of different groups was subcutaneously injected with 0.1 ml of 20% suspension of SRBC into the right hind footpad. The contralateral paw also received an equal volume of normal saline. The administration of experimental samples (normal saline, levamisole and *E. tirucalli* extract) were continued until the 14th day. On the 14th day, the animals were again subcutaneously injected with 0.1 ml of SRBCs into the left hind footpad of the mice. The extent of delayed-type hypersensitivity (DTH) response in the mice was determined by measuring the footpad thickness 24 h of challenge using a slide calipers. The difference in the thickness of the right hind paw and the left hind paw was then used as a measure of delayed type hypersensitivity reaction and was expressed as a mean percent increment in thickness/edema.

Molecular docking

Protein preparation

Protein models PDB ID: 6COX (COX II), 6R3Q (adenyl cyclase), 1HXM (T cell) were downloaded from the protein data bank (https://www.rcsb.org/). After downloading, those proteins were modified by Discovery Studio 2020 client to remove all the attached water and associated ligands. This was done to make the respective protein available for further attaching with selected ligands in molecular docking [10].

Ligand preparation

At first, we have listed some compounds that are reported to be present in this plant by reviewing literatures especially in the latex and leaves portion as we have conducted all of our laboratory experiments with this extract. 3D structures of some ligands were downloaded from Pubchem site such as 12-deoxyphorbol esters (CID: 119252), ingenol (CID: 442042), 4-deoxyphorbol (CID: 157479), tri-methyl ellagic acid (CID: 8357), taraxasterol (CID: 115250), tirucallol (CID: 101257) etc. Using chemdraw software, we have designed 3D structures of cycloeuphordenol, cyclotirucanenol, tirucalicine as their 3D structures were not found in Pubchem site (https://pubchem.ncbi.nlm.nih.gov/). We also designed 3D structures of *β*-sitosterol, *β*-amyrin and 12-deoxy-4*β*-hydroxyphorbol-13- phenyl acetate-20-acetate but those were not processed in further docking process conducted in PyRx. We have also downloaded 3D structures of standard drugs such as diclofenac Na (CID: 3033), bisacodyl (CID: 2391) and levamisole (CID: 26879) from Pubchem. Energy of all of these ligand molecules were minimized using PyRx [10].

Site-specific binding and visualization

We had decided to conduct blind docking so that every part of the protein molecules might be available for binding. It was carried out in PyRx and auto dock vina 4.2. The grid dimensions for 6COX, 6R3Q, 1HXM were 60.3403:78.4550:68.8632, 132.8413:67.5460:95.6531 and 37.7845:77.9791:60.1229, respectively. Site-specific molecular docking was conducted in above mentioned software and finally 3D interactions of the protein-ligand binding were processed in Pymol [11].

Statistical Analysis

All experimental data presented in this article are expressed as mean = (number ± standard deviation (STD)). Using Statistical Package for the Social Sciences (version 25), we had analyzed the data by one-way ANOVA followed by Tukey as post hoc test. In all cases, the statistical significance was considered as $p \leq 0.05$. Graphical presentations were designed by Graph pad Prism software (version 21) [12].

Results

In the phytochemical assessment, *E. tirucalli* extract revealed the presence of various important types of phytochemical groups like reducing sugars, tannins, flavonoids, alkaloids, gums, glycosides, terpenoids, steroids etc.

No mice were found dead in the acute toxicity test up to 4th days. All of them were found normal. So, 50% lethal dose could not be calculated. For that reason, we decided to conduct further tests on mice using this extract at 200 and 400 mg/kg dose as these were found safe to them.

In determining total content of different secondary metabolites in *E. tirucalli* extract, we have estimated their values in and the values are presented in Table 1. Calibration curves for the respective standards are presented in figure 2.

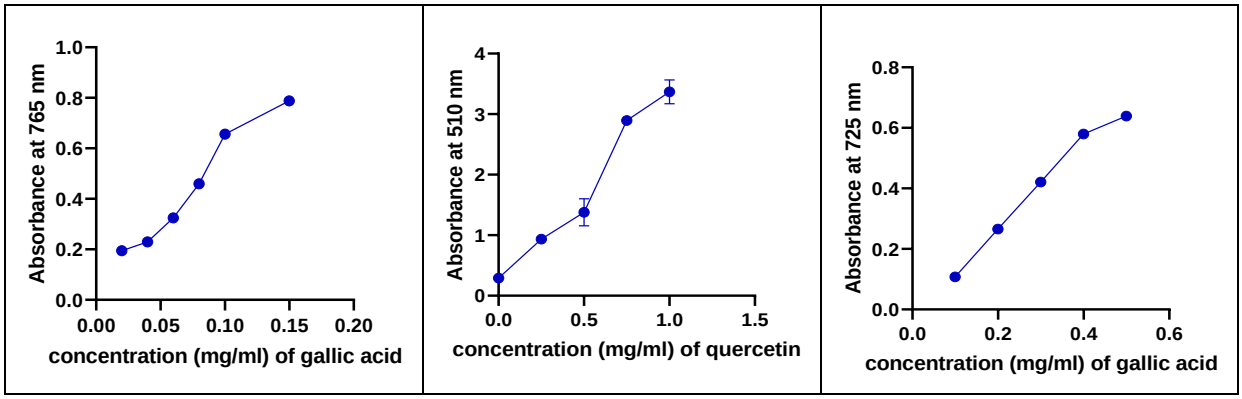


Fig 2: Calibration curve of gallic acid for estimation of total phenolic content, quercetin for estimation of total flavonoid content and gallic acid for estimation of total tannin content in *E. tirucalli* extract.

Table 1: Approximate secondary metabolites content in *E. tirucalli* extract

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg GAE/g)
E. tirucalli extract	111.4	105.94	67.1

In the acetic acid-induced writhing model, *E. tirucalli* extract significantly lessened the writhing reflexes in mice upto 42.22% and 65.18% in 200 and 400 mg/kg doses, respectively, where diclofenac Na reduced writhing 78.51% in 25 mg/kg dose (Table 2).

Table 2: Effect of *E. tirucalli* extract on acetic acid-induced writhing in mice

Treatment group	Dose	Mean writhing ±SD	% Inhibition of writhing
Negative control (1% tween 80)	10 ml/kg	27 ± 2 ᵉ □ ■	---
Standard (diclofenac Na)	25 mg/kg	5.8 ± 0.84 * □ ■	78.51
E. tirucalli extract	200 mg/kg	15.6 ± 1.52 * ᵉ ■	42.22
E. tirucalli extract	400 mg/kg	9.4 ± 11.40 * ᵉ □	65.18

Data are means of five replicates ± SD;
* $p < 0.05$ vs. negative control (Dunnett's t-test)
ᵉ $p < 0.05$ vs. diclofenac Na 25 mg/kg
□ $p < 0.05$ vs *E. tirucalli* extract 200 mg/kg.
■ $p < 0.05$ vs. *E. tirucalli* extract 400 mg/kg
(pair-wise comparison by Post Hoc Tukey test).

In the laxative test, stool weight (gm) of mice groups of *E. tirucalli* extract at both doses were found quite increased comparing with the mice of negative control group (29.31% and 63.79% in 200 and 400 mg/kg doses, respectively). Stool weight of mice groups treated with bisacodyl at 10 mg/kg was increased up to 108.62% (Table 3).

Table 3: Effect of *E. tirucalli* extract on laxative test

Treatment group	Dose	Mean stool weight (gm) ±SD	% Increase in stool weight
Negative control (1% tween 80)	10 ml/kg	0.58 ± 0.02 ᵉ □ ■	---
Standard (bisacodyl)	10 mg/kg	1.21 ± 0. 03* □ ■	108.62
E. tirucalli extract	200 mg/kg	0.75 ± 0.01 * ᵉ ■	29.31
E. tirucalli extract	400 mg/kg	0.95 ± 0.02 * ᵉ □	63.79

Data are means of five replicates ± SD;
* $p < 0.05$ vs. negative control (Dunnett's t-test)
ᵉ $p < 0.05$ vs. bisacodyl 10 mg/kg
□ $p < 0.05$ vs *E. tirucalli* extract 200 mg/kg
■ $p < 0.05$ vs. *E. tirucalli* extract 400 mg/kg
(pair-wise comparison by Post Hoc Tukey test).

In the delayed type hypersensitivity test, mice treated with *E. tirucalli* extract showed an extension or edema in their treated paws (left) comparing to their left paws. The paw diameter and their percent increase are plotted in table 4. Here we can see that, after 24 hours of the experiment to immune response to SRBC, mice treated with *E. tirucalli* extract at 400 mg/kg dose revealed the increase in paw diameter upto 17.74%. And for levamisole, it was 27.53% (Table 4).

Table 4: Effect of *E. tirucalli* extract on delayed type hypersensitivity in mice

Treatment group	Dose	Mean paw diameter (mm) ± SD of right foot	Mean paw diameter (mm) ± SD of left foot	% Increase in paw thickness
Control (1% tween 80)	10 ml/kg	5 ± 0.7	5.3 ± 1.0 ᵉ ■	5.66
Negative control (0.9% NaCl solution)	10 ml/kg	5 ±,0.2	5.5 ± 0.255 ᵉ □■	9.09
Standard (levamisole)	50 mg/kg	5 ± 0.122	6.9 ± 0.212 ω * □ ■	27.53
E. tirucalli extract	200 mg/kg	5 ± 0.187	5.8 ± 0.141 ω *,■	13.79
E. tirucalli extract	400 mg/kg	5.1 ±,0.234	6.1 ± 0.2915 ω * ᵉ □	18.03

Data are means of five replicates ± SD
ω $p < 0.05$ vs. control (Dunnett's t-test)
* $p < 0.05$ vs. negative control
ᵉ $p < 0.05$ vs. levamisole 50 mg/kg,
□ $p < 0.05$ vs *E. tirucalli* extract 200 mg/kg,
■ $p < 0.05$ vs. *E. tirucalli* extract 400 mg/kg (pair-wise comparison by Post Hoc Tukey test).

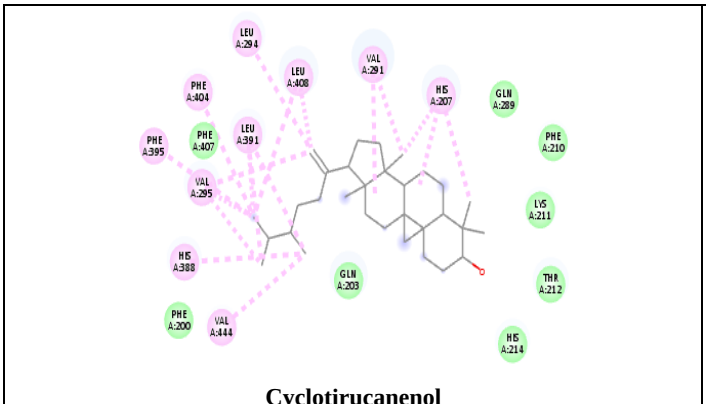
In the molecular docking, selected ligand molecules were docked with different proteins. The docking score (binding affinities) of the ligands with the 6COX, 6R3Q and 1HXM are listed in table 5.

Table 5: Docking score (kcal/mol) of ligands with different proteins

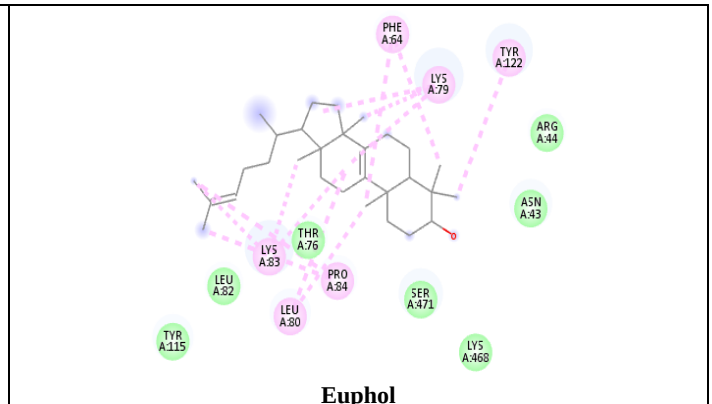
Name of the compound	Pubchem ID	Proteins		
		6COX	6R3Q	1HXM
12-Deoxyphorbol	119252	−7.3	−7.1	−6.1
Ingenol	442042	−7.6	−7.2	−6.2
Euphol	441678	−8.2	−8.6	−7.2
4-deoxyphorbol	157479	−7.5	−7.1	−6.4
Cycloeuphordenol	-----	−7.7	−7.0	−6.0
Cyclotirucanenol	-----	−8.1	−8.0	−6.5
Tirucalicine	-----	−7.0	−6.8	−6.2
Tri-methyl ellagic acid	8357	−6.3	−5.2	−5.1
Taraxasterol	115250	−9.1	−8.9	−7
Tirucallol	101257	−8.1	−8.8	−7.2
Diclofenac Na	3033	−6.5		
Bisacodyl	2391		−7.4	
Levamisole	26879			−4.8

[Compounds showing best binding affinities (<–8 kcal/mol) are marked in bold style]

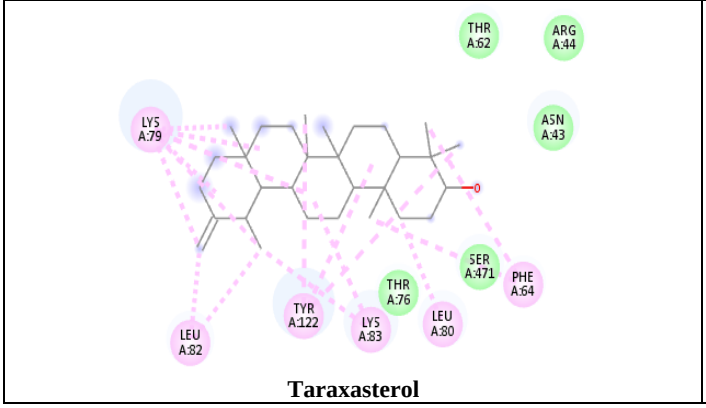
In case of binding with 6COX protein, 4 ligands namely cyclotirucanenol, euphol, taraxasterol and tirucallol revealed notable binding affinities (<–8 kcal/mol). So, we further then tried to find out their 2D and 3D interactions with the 6COX protein (presented in figure 3 and 4, respectively). The interacting amino acids are listed in table 6.



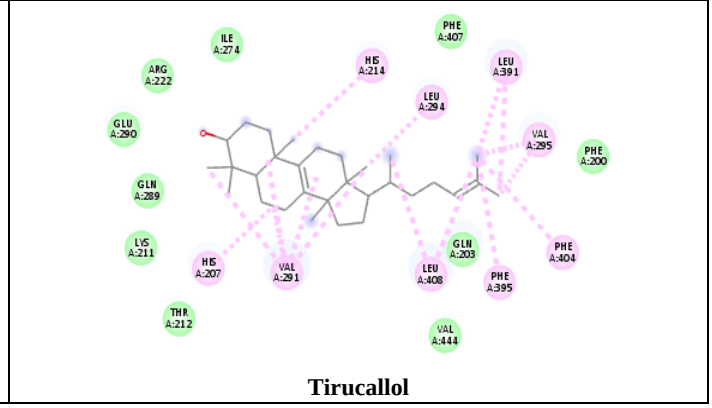
Cyclotirucanenol



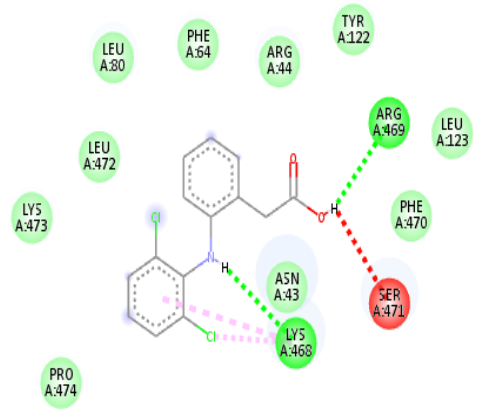
Euphol



Taraxasterol



Tirucallol



Diclofenac Na

Interactions

Van der Waals	Alkyl/ Pi-alkyl
Carbon Hydrogen Bond	Pi-Sigma
Conventional Hydrogen bond	Unfavorable Donor-Donor

Fig 3: 2D interactions of amino acids of 6COX proteins with cyclotirucanenol, euphol, taraxasterol, tirucallol and diclofenac Na

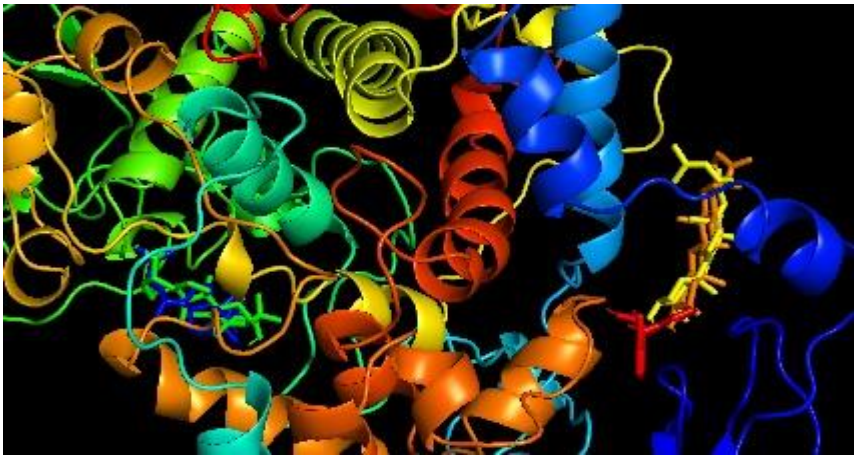
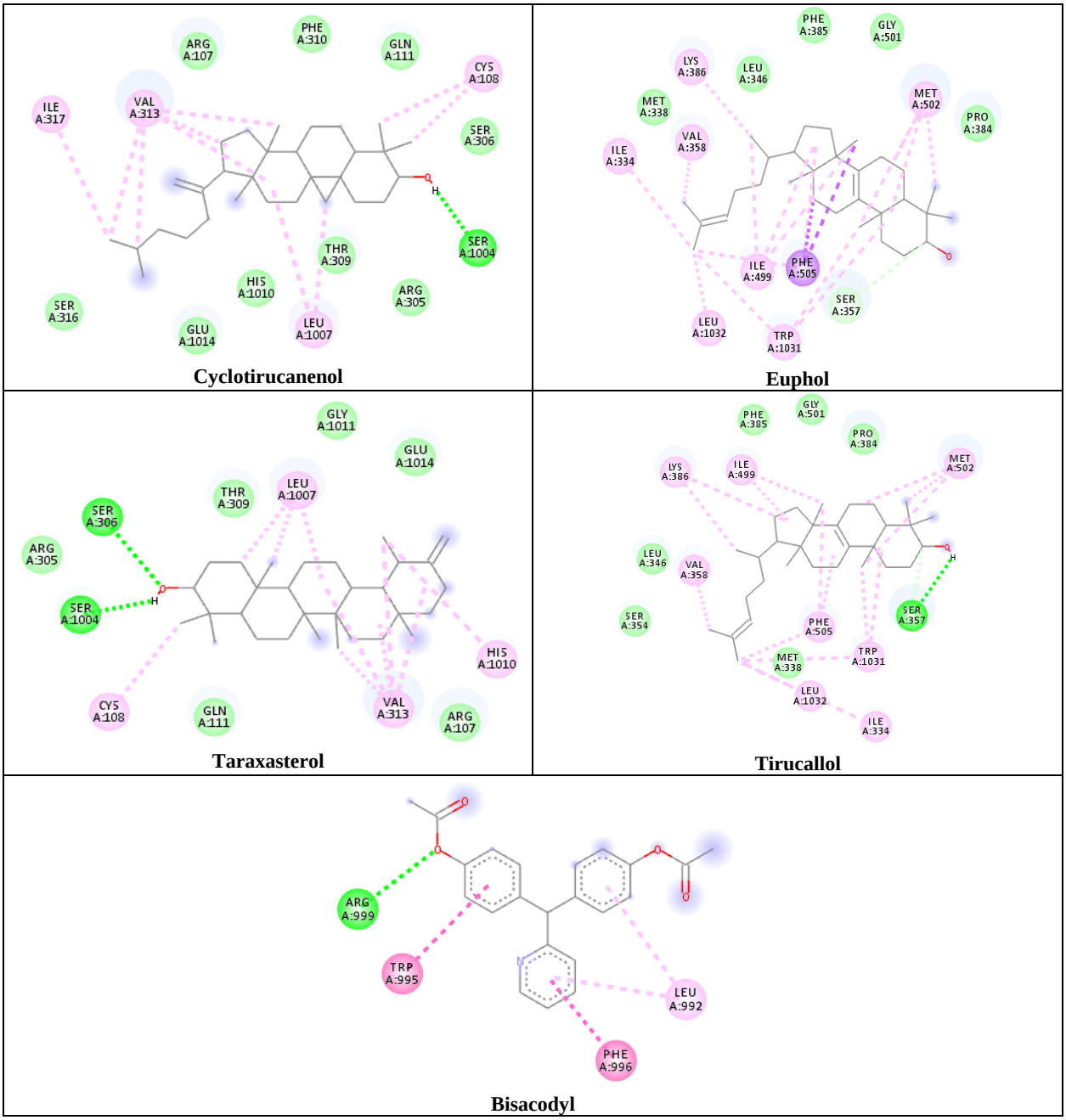


Fig 4: Binding pocket of cyclotirucanenol (green), euphol (yellow), taraxasterol (orange), tirucallol (blue) and diclofenac Na (red) with 6COX protein

Table 6: Binding of different ligands with amino acids of 6COX protein

Name of the ligand	Interacting amino acids
Cyclotirucanenol	Gln203, Gln289, Phe200, Phe210, Phe395, Phe404, Phe407, Lys211, Thr212, His207, His214, His388, Val391, Val295, Val444, Leu394, Leu391, Leu408
Euphol	Tyr115, Tyr122, Arg4, Asn43, Ser471, Lys79, Lys83, Lys468, Pro84, Thr76, Leu80, Leu82, Phe64
Taraxasterol	Lys79, Lys83, Leu80, Leu82, Tyr122, Thr62, Thr76, Ser471, Phe64, Asn43, Arg44
Tirucallol	His207, His214, Leu294, Leu391, Leu408, Phe407, Phe200, Phe395, Phe404, Gln203, Val291, Val295,Val444, Thr212, Lys211, Gln2289, Glu290, Arg222, Ile274
Diclofenac Na	Arg469, Leu123, Leu472, Phe64, Phe470, Ser471, Asn43, Lys468, Lys473, Pro474, Arg44, Tyr122

In case of binding with 6R3Q protein, again those above 4 ligands revealed notable binding affinities (<–8 kcal/mol). So, we further then tried to find out their 2D and 3D interactions with the 6R3Q protein (presented in figure 5 and 6, respectively). The interacting amino acids are listed in table 7.



Interactions

Van der Waals	Alkyl/ Pi-alkyl
Carbon Hydrogen Bond	Pi-Sigma

Figure 5: 2D interactions of amino acids of 6R3Q proteins with cyclotirucanenol, euphol, taraxasterol, tirucallol and bisacodyl

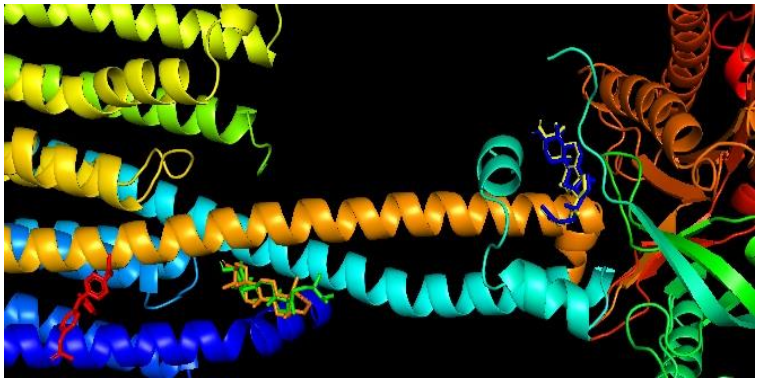


Fig 6: Binding pocket of cyclotirucanenol (green), euphol (yellow), taraxasterol (orange), tirucallol (blue) and bisacodyl (red) with 6R3Q protein

Table 7: Binding of different ligands with amino acids of 6R3Q protein

Name of the ligand	Interacting amino acids
Cyclotirucanenol	Cys108, Ser306, Ser316, Ser1004, Arg107, Arg305, Thr309, His1010, Leu1007, Glu1014, Ile317, Val313, Phe310, Gln111
Euphol	Gly501, Met 338, Met502, Pro384, Ser357, Phe385, Phe505, Ile334, Ile499, Trp1031, Leu346, Leu1032, Lys386,
Taraxasterol	His1010, Arg107, Arg305, Val313, Gln111, Cys108, Ser306, Ser1004, Thr309, Leu1007, Gly1011, Glu1014
Tirucallol	Ser354, Ser357, Phe385, Phe505, Trp1031, Met338, Met502, Leu346, Leu1032, Ile334, Ile499, Val358, Lys386, Gly501, Pro384
Bisacodyl	Leu992, Phe996, Trp995, Arg999

In case of binding with 1HXM protein, none of the ligands revealed notable binding affinities (<–8 kcal/mol). So, we did not try to find out any interactions with this protein.

Discussion

Traditional medicine is one of the oldest healthcare system by human. It has many significant contributions to modern advanced medical system. Still traditional medicine is very much popular in many countries. Plants are the main source of medicinal components in traditional medicine as well as modern allopathic system. Moreover, people from most of the third world countries depend on herbal medicine as they cannot afford the uprising treatment cost. Due to containing different types of important bioactive phytochemicals, medicinal plants are always become an attention to natural product scientists. Although modern allopathic system have tremendous improvement over the last decades, still almost 80% of modern allopathic medicine are directly obtained from various medicinal plants. That's why researchers always give their importance contribution to derive more and more better plant derived drug molecules.

Antioxidants are the mostly important and available plant derived compounds having numerous type of medicinal importance. Almost all plants synthesize antioxidant for its own growth, maturation, reproduction, protection from viruses and bacteria. But, due to the inability to synthesize antioxidants, human and other animals have to rely on plants. Antioxidants are well reported for their neutralizing or scavenging capability of different free radicals and thus they protect us from their associated diseases like cancer, stroke, diabetes, atherosclerosis, neurological, cardiac and renal impairment [13]. Mostly plant derived antioxidants are the large group of different polyphenols, flavonoids and tannins. The ability of polyphenols to scavenge free radicals is widely recognized, as are their strong anti-aging, cardioprotective, neuroprotective, hepatoprotective, anti-inflammatory, antimicrobial, and antiproliferative properties [14]. Flavonoids have been shown to exhibit a variety of biological activities, including central vascular effects, anti-inflammatory, hepatoproective, antitumor, antimicrobial, and antiviral properties [15]. Tannins are used to treat gum inflammation, minor burns, hemorrhoids, varicose ulcers, and frostbite [16]. The plant *E. tirucalli* is already reported for its different free radical properties. In a manuscript, Chanda and Baravalia, 2010 conducted DPPH, hydroxyl radical and superoxide radical scavenging properties of this plant [17]. So, we decided to estimate different antioxidant secondary metabolite content like phenols, flavonoids and tannins from this extract. From our experiment, we can say that, this extract have some, antioxidant compounds which can be useful to reveal different types of therapeutic benefits.

Pain is one of the most common pathologic condition being suffered by many persons in different situations. Due to both extracellular and intracellular stimulatory perceptors, it always produces an unpleasant feeling. Prostaglandins (PGI_2, PGE_2, $PGF_{2\alpha}$), cytokines, and leukotrienes are released by the phospholipids in the afflicted tissues upon the release of arachidonic acid. These substances cause the perception of pain sensations such as anxiety, sweating, nausea, palpitations, and abnormal variations in blood pressure. Mice experience pain when acetic acid is administered intraperitoneally because it triggers physiological reactions that cause the cyclooxygenase pathway to synthesize different pain mediatory prostaglandins and cytokines [18]. From our acetic acid-induced writhing model test in mice, we observed that, *E. tirucalli* extract significantly reduced the no. of writhing at both 200 and 400 mg/kg doses at dose dependent manner. Phytochemicals like alkaloids, glycosides, terpenoids, polyphenols, flavonoids might be responsible for this analgesic and anti-inflammatory effects as their such properties have already been reported [19].

Constipation is one of the major gastrointestinal problem that is due to stool hardening. During the stooling process, patients occasionally experience excruciating pain. Low water intake, intake of foods having no or few fiber and lack of physical exercise may cause this problem. Prolonged constipation may increase the risk of developing piles, enlarged hemorrhoids, and colorectal cancer, among other colorectal conditions [20]. Laxatives are commonly used to treat constipation because they make stools easier to pass and add bulk to the intestinal contents by holding onto water [21]. In the laxative test, we observed that mice treated with *E. tirucalli* extract showed increase in excreting stool comparing with mice of negative control group. This plant have traditional use as purgative and our result also signifies its uses. The laxative effect of this extract might be due to the presence of carbohydrates, glycosides, tannins, flavonoids which have reported laxative effect [20].

The recruitment of T cells into tissues to be activated by antigen-presenting cells and produce cytokines that mediate local inflammation is known as delayed type hypersensitivity. It is now understood that CD8+ T cells mediate responses in autoimmune diseases, drug eruptions, asthma, and allergic contact dermatitis. There is now considerable evidence that diseases may be mediated by CD8+ cells. External antigens are phagocytosed and processed so that they can be presented on Major Histocompatibility Complex molecules. This process are very much important to destroy any kind of foreign bodies to protect body's own cells. In the delayed hypersensitivity test, mice treated with *E. tirucalli* extract showed significant increase in the paw diameter that determines the hypersensitivity reactions. This property might be helpful to strengthen our body's own defensive mechanism by further phagocytosis process to kill the harmful foreign particles [22].

After get well response from the biological tests, we decided to conduct molecular docking analysis of different reported compounds from this extract with respective protein models.

An established method of computational modeling called "molecular docking" describes how molecules should be arranged or oriented with respect to particular proteins when those proteins are combined to form a stable complex. Molecular docking suggests that a compound's activities increase with its affinity for proteins. Characterizing binding

behavior is essential for both logical drug design and understanding fundamental biological processes [23].

6COX is a protein of the COX-II enzyme. The primary mediators of peripheral pain, inflammation, and pyrexia are the COX enzymes, which also release arachidonic acids and other chemicals like prostaglandins and leukotrienes. In case of molecular docking of ligands with the 6COX proteins, we have found 4 ligands namely cyclotirucanenol, euphol, taraxasterol and tirucallol revealed notable binding affinities (<–8 kcal/mol). From their 2D interaction figure, we could not find a single amino that is common among all of the ligands. In most of the cases, the ligands bound with amino acids by Van Der Waals and Pi-alkyl bonds. Some of them were conventional hydrogen bond and Pi-sigma bond too. On the other hand, from their 3D interaction figure, we have found that, euphol and taraxasterol were bound in quite close area like diclofenac Na got bound. Cyclotirucanenol and tirucallol got bound to a different region. From these results, we can summarize that, although these 4 ligands did not bound to a same region, but they have notable impact in binding with the 6COX protein to reveal analgesic and anti-inflammatory response.

In case of molecular docking of laxative test, we have selected a protein PDB ID: 6R3Q (a protein of adenyl cyclase) as most of the laxative drugs get bind with the adenyl cyclase to express their effect. Like docking with 6COX protein, the same 4 ligands revealed notable binding affinities (<–8 kcal/mol). From their 2D interaction figure, we could not find a single amino that is common among all of the ligands. In most of the cases, the ligands bound with amino acids by Van Der Waals and Pi-alkyl bonds. On the other hand, from their 3D interaction figure, we have found that, euphol and tirucallol were bound in quite close area while cyclotirucanenol and taraxasterol got bound to a close area in different region. None of these 4 ligands got bound in close area like bisacodyl got bound. From these results, we can summarize that, although their binding regions are different, but they are capable in binding with the 6R3Q protein to reveal the desired laxative effect.

In case of molecular docking of delayed hypersentivity test, we have selected a protein PDB ID: 1HXM (a protein of T cell receptor), as the T cells are mostly involved in eliciting hypersensitivity reaction. But, none of the ligands showed notable binding affinities with these protein. But in case of *in vivo* analysis, we have found that, *E. tirucalli* extract showed notable hypersensitivity reaction. These *in vivo* effect might be due to binding with any other proteins of T cell. Further docking analysis may reveal the secret in eliciting hypersentivity response from this plant.

Conclusion

Euphorbia tirucalli is a quite common medicinal in the world from Euphorbiaceae family. It has many therapeutic importance in traditional medicine. Our present study was conducted considering its uses in treating pain and inflammation and its uses as purgative and also immunomodulatory effects. From our preliminary analysis, we can say that this plant is enriched with numerous antioxidant groups that might be helpful in combating many diseases. This extract showed good responses in analgesic, anti-inflammatory, laxative and immunomodulatory tests. Molecular docking analysis also revealed the effectiveness of 4 compounds in eliciting anti-inflammatory and laxative effects. These results also justifies its traditional uses. We hope, our results will be helpful in conducting further researches on this plant to isolate newer and better lead molecules in future.

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Conflict of interest

There are no confliction of interest among the authors.

Author's declaration

The authors declare that all of the experimental data presented here are original and all types of liabilities for claiming any content of this manuscript will be fully borne by them.

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