In-Vivo AND In-Silico ANALYSIS OF ANTI-INFLAMMATORY, ANTIPYRETIC AND ANALGESIC ACTIVITY OF METHANOLIC SEED EXTRACT OF Citrus Colocynthis

AREEJ KHALID [1], NUREEN ZAHRA [1], IQRA MUSHTAQ [1], BASIT ZEESHAN [2], ABID SARWAR [1], AMMENA Y. BINSALEH [3], NAWAL AL-HOSHANI [4], MAHER S. ALWETHAYNANI [5], AREEJ A ALHHAZMI [6], FAKHRIA A. AL-JOUFI [7], AND TARIQ AZIZ [8]*

[1] *Institute of Molecular Biology and Biotechnology, the University of Lahore Punjab Pakistan.*
[2] *Institute of Industrial Biotechnology, GC University Lahore Punjab Pakistan.*
[3] *Department of Pharmacy Practice, College of Pharmacy, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia.*
[4] *Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia.*
[5] *Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Alquwayiyah, Riyadh, Saudi Arabia.*
[6] *Clinical Laboratory Sciences Department. Applied Medical Sciences, Taibah University, Medina, Saudi Arabia.*
[7] *Department of Pharmacology, College of Pharmacy, Jouf University, 72341 Aljouf, Saudi Arabia.*
[8] *Laboratory of Animal Health, Food Quality and Hygiene, University of Ioannina Arta Greece.*

ABSTRACT

Ongoing investigations into the bioactive components of therapeutic plants are increasingly supported by the integration of advanced technological tools. The aim of the current study was to analyze anti-inflammatory, antipyretic and analgesic activities from methanolic seed extract of *Citrullus colocynthis* (bitter melon) by using different *in vitro* and *in vivo* experimental models in albino rats. For extract preparation, dried seeds were soaked in methanol for two weeks, followed by filtration and solvent evaporation to yield concentrated extract rich in bioactive compounds. The anti-inflammatory effects were evaluated using carrageenan-induced paw edema and cotton pellet granuloma models in albino rats, where the extract achieved up to 100% inhibition. Antipyretic activity was assessed through a Brewer's yeast-induced pyrexia model, showing 86% inhibition, while analgesic activity was measured using tail immersion and acetic acid-induced writhing methods, with the extract producing 100% pain inhibition. The extract demonstrated comparable efficacy to standard drugs such as phenylbutazone, diclofenac, and paracetamol. These pharmacological results were further supported by histological and biochemical analyses, which confirmed significant reductions in inflammatory and pain-related responses. In addition, *in silico* docking studies were conducted to explore the binding interactions of key phytochemicals alpha-spinosterol, ascorbic acid, and chlorogenic acid with three target proteins involved in inflammation and pain pathways: PTGS2 (COX-2), TLR2, and TRPV4. The phytochemicals showed strong binding affinities and hydrogen bonding patterns similar to standard drugs. For example, alpha-spinosterol and diclofenac exhibited hydrogen bonding with PTGS2 residues ARG311 and ASP314, while chlorogenic acid and diclofenac bound effectively to TRPV4 residues ASN361 and ARG392. These findings aligned with the traditional use of *Citrullus colocynthis* in treating digestive disorders, fever, and inflammatory conditions. The extract potent biological activities and comparable performance to reference drugs support its potential as a promising natural therapeutic agent. However, further research is needed to determine its optimal dosage, safety profile, and clinical applicability. The study underscores the future potential of *Citrullus colocynthis* not only in medicine but also as an eco-friendly bio-agent in agricultural applications.

Keywords: *Citrullus colocynthis, phytochemicals, therapeutic properties, pharmacological properties, medicinal uses*.

INTRODUCTION

Medicinal plants have been used in healthcare for centuries, and their application in the prevention and treatment of diseases continues to grow globally (Abid *et al.,* 2025; Kiran *et al.,* 2025; Zahra *et al.,* 2025; Afsar *et al.,* 2024; Aziz *et al.,* 2024; Ejaz *et al.,* 2024; Farah *et al.,* 2024; Li *et al.,* 2022). Bitter fruits have played a significant role in human history, shaping cultural practices, artistic expressions, and culinary traditions since ancient times. The bitter apples were served in early times by ancient people. Bitter fruits were often used in medicinal preparations, as they were believed to possess many healing properties. A very sour apple or colocynth was one such botanical medicine used in ancient times; its fruit and root extracts were employed to treat illnesses such as indigestion, various diseases, and skin conditions (Rao *et al.,* 2023; Caesar T., 2022; Behera *et al.,* 2021) Bitter Apples have played a significant role in traditional medicinal systems worldwide. Ancient healers and herbalists utilized bitter fruits for their purported medicinal properties, often as digestive aids, cathartics, or remedies for various ailments (Mars *et al.,* 2024). Flavonoids, alkaloids and other phytoconstituents of bitter fruit have been identified for their inhabitation action against glycogenesis pathways in the body. Moreover, terpenoids have been observed for their antioxidant; anti-inflammatory and anti-diabetic potential in experimental models (Alam *et al.,* 2022).

Citrullus colocynthis belong to the family Cucurbitaceae commonly called as the bitter apple or colocynth; a plant that flourishes in dry regions from the Mediterranean Sea to the North of Africa and the Middle East. It has creepers where leaves are cut into deep tubes. It fetches small, sour fruits that are just similar yellow-greenish melon. Bitter apple tree bears small yellow flowers which differs from any known flower arrangement. They are arranged in singles or in clusters at the different nodes of the vine like structure of the plant (Choudhary *et al.,* 2023; Al-Snafi AE 2021; Dehgan Bijan 2023). Traditionally used in numerous cultures for the remedy of illnesses consisting of inflammation, fever, and ache, this plant has garnered big interest inside the area of phytomedicine. Despite its historical applications, scientific validation of its therapeutic capacity stays critical. *Citrullus colocynthis* has all unique therapeutic properties to possess antidiabetic, hypolipidemic, antineoplastic, antioxidant, anti-inflammatory, profibrinolytic, analgesic, antiallergic, antimicrobial, pesticidal, immunostimulant activity. It also affects the reproductive health and fertility. Based on this review of literature, it can be concluded that *Citrullus colocynthis* possesses multiple pharmacologic effects with a noticeable emphasis on the antidiabetic effect (Li *et al.,* 2022; Meybodi MSK 2020).

A class of potent biocatalysts include cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which catalyse reactions involved in the production of inflammatory mediators such as prostaglandins and leukotrienes. Bitter apple extracts have been proved to suppress the activity of these enzymes, and this process leads to the decrease of inflammation (Zhang *et al.,* 2018). In analgesic activities, Firstly, these compounds may suppress the generation or activity of mediators including cytokines and prostaglandins which itself plays a significant role in transmitting pain signals secondly bitter apple may alter the firing and sensitivity of neurotransmitters associated with pain perception. There are some active components of bitter apple extracts that have been reported with strong effect on neurotransmitter receptors within the central nervous system, including opioid receptors or GABA receptors, which are involved in pain regulation (Forouzanfar *et al.,* 2019). Perhaps the most probable route at which bitter apple might be able to express its antipyretic effect is through its analgesic effect. Although, it has some bioactive components verified for anti-inflammatory properties in experiments conducted on animal models and cell lines. As a result, bitter apple is able to decrease the level of the mediators and pathways responsible for inflammation commonly seen in fever and contributing to temperature regulation (Hameed *et al.,* 2020). A specific part of the bitter apple (*Citrullus colocynthis*) may influence immune cell activity and cytokine

*Corresponding author email: iwockd@gmail.com

production, potentially helping to regulate the immune response. The present study aims to bridge this gap by conducting comprehensive *in vivo* and *in silico* analyses to evaluate the anti-inflammatory, antipyretic, and analgesic activities of the methanol extract of *Citrullus colocynthis* (Zhang *et al.,* 2021).

MATERIALS AND METHODS

Sample Collection

Dried Bitter Apple Seeds or more commonly known as 'Kali Jheenga' were purchased from local market in Anarkali Bazar Lahore during July 2023 and their scientific identification and authentication of the dried seeds were initially conducted at the Department of Life Sciences, Lahore University of Management Sciences (LUMS), and subsequently verified at the Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan. These seeds were clean physically and kept at room temperature in a dry place before start of experimentation.

Extract Preparation

Extract of seeds

For preparation of extract, collected seeds were soaked in measured quantities of methanol containing blue capped glass bottles. The mixture was shaken vigorously for 2-3 minutes and then kept at room temperature for two weeks to allow the methanol to extract bioactive compounds from the seeds. The mixture was filtered through Whattman filter paper to remove solid residues after 14 days of incubation, and supernatant was poured into petri dishes. The petri dishes were left at room temperature for 10 days for evaporation of methanol and collection of dried extract. The final samples were collected into Eppendorf tubes and labelled for identification and further analysis Hameed *et al.,* 2020).

Experimental rats

The pharmacological activities including anti-inflammatory, analgesic, and antipyretic effects were evaluated using albino rats of either sex. These rats were obtained from the University of Lahore (UOL) and housed in the UOL animal facilities under standard laboratory conditions in individually labelled cages. The animals were divided into three experimental groups, with each group consisting of four rats per cage. Prior to the commencement of the experiments, all rats underwent a fasting period to ensure metabolic uniformity. After fasting, the animals were given free access to clean drinking water and a nutritionally balanced diet.

Measurement of anti- inflammatory activity

Carrageenan at concentration of 50, 100, 200, and 400 mg/kg was applied to cause inflammation, and this was prevented by diclofenac and *Citrullus colocynthis* extract. A total 24 albino rats were categorized into three groups where group 1 treated with Normal saline as control group. The standard drug diclofenac was administered to group 2 via the paw at four different concentrations: (50,100, 200 and 400) mg/kg. The third group experimental rats were treated with the methanolic seed extract of *Citrullus Colocynthis*, prepared previously in the laboratory. For inflammation induction different concentrations of carrageenan (50, 100, 200, and 400) mg/kg were given to the rats of all groups following body weight criteria of animals. The carrageenan concentrations were injected to the sole to make inflammation in hind paw. The paw size was recorded every hour for four hours and higher swelling observed in the respective time. A day later paw size was normal [22]. After induction of inflammation the control group was treated with distilled water or normal saline while in standard group rats, diclofenac was administered by injection into the sole. In the experimental group, the rats were treated with different concentrations (50, 100, 200, and 400) mg of *Citrullus Colocynthis* methanolic seed extract and the anti- inflammatory activity was measured by this formula:

$$\% \text{ Inhibition} = \frac{(\text{Ct-Co}) \text{ control} - (\text{Ct-Co}) \text{ treated} \times 100}{(\text{Ct-Co}) \text{ control}}$$

Where,

Co = Reading of paw before carrageenan,

Ct = Volume of the hind paw after carrageenan

(Ct-Co) = Volume of the hind paw of the treated group after carrageenan injection

Measurement of anti- pyretic activity

Albino rats were divided into three groups, with each cage housing four rats. Fever was induced in the rats using Brewer's yeast mixed with distilled water at concentrations of 50, 100, 200, and 400 mg. Group 1 rats were treated with normal saline or distilled water while rats in group 2 and 3 were treated with different concentrations of standard drug, diclofenac, at doses of 50, 100, 200, and 400 mg and *Citrullus Colocynthis* seed extract in methanol previously prepared in the laboratory in varying concentrations. The yeast was injected into the neck, causing hyperpyrexia in the rats after 20 hours. The maximum observed temperature was 103°F, measured with a digital thermometer inserted into the rats' colons. Under controlled pH conditions for 18 h their body temperatures were again recorded; animals with body temperatures ≥ 100.4°F were selected for testing. Temperature measurements were taken hourly, three to four times (Dehgan Bijan 2023). The antipyretic activity was calculated by the given formula:

$$\% \text{ reduction} = \frac{B - Cn}{B - A} \times 100$$

Here, B = Temperature after injecting Yeast (fever-induced), A = Temperature before injecting yeast (original body temperature), Cn = Body Temperature after 3 hours of fever induction.

Measurement of analgesic activity

The analgesic activity of the methanolic seed extract of *Citrullus colocynthis* was evaluated using the acetic acid-induced writhing method in albino rats. This model assesses the animals' muscular response to chemically induced pain through abdominal constrictions and hind limb extensions. Albino rats weighing 25–30 g were used, and the animals were randomly assigned into three main groups: a control group, a standard drug group, and a test group. Each group was further subdivided to receive one of four treatment doses 50, 100, 200, or 400 mg/kg body weight administered orally.

The animals were fasted for 16 hours prior to the experiment while maintaining access to water. One hour after administration of the *Citrullus colocynthis* seed extract, and 30 minutes after administration of the standard drug (diclofenac), pain was induced by intraperitoneal injection of 0.2 mL of a 3% acetic acid solution. The number of writhing responses characterized by abdominal contractions and hind limb stretching was recorded over a 20-minute observation period beginning 5 minute's post-injection. The standard drug group received diclofenac (150 mg/kg) as a positive control, while the control group received only normal saline. Analgesic activity was quantified by comparing the number of writhes in the test and standard groups to that of the control group, using the following formula:

$$\text{Analgesic activity} = \frac{Nt - Nc}{Nc} \times 100$$

Here, Nc= Writing on the control group and Nt = Writing of Treated Group

Computational analysis

Preparation of proteins

The *in-silico* evaluation of the anti-inflammatory, analgesic, and antipyretic activities of *Citrullus colocynthis* seed extract was carried out using a range of bioinformatics tools, including ChemSketch, UCSF Chimera 1.15, PyMOL, PyRx, and Discovery Studio. Three target proteins involved in inflammatory and pain pathways PTGS2 (Cyclooxygenase-2), TLR2 (Toll-like receptor 2), and TRPV4 (Transient Receptor Potential Vanilloid 4) were selected for docking studies.

The three-dimensional (3D) crystal structures of these proteins were retrieved from the Protein Data Bank (PDB) in PDB file format. Using UCSF Chimera, non-essential elements such as co-crystallized ligands, water molecules, and ions were removed to avoid interference in the docking process. All chains except one were deleted to isolate a single protein chain, which was then saved in PDB format for further analysis. Secondary structure elements including α-helices, β-sheets, and coils were evaluated using the VADAR (Volume, Area, Dihedral Angle Reporter) server to assess structural integrity. Additionally, the quality of

the refined protein models was validated by Ramachandran plot analysis using WinCoot, providing information on the stereochemical conformations of amino acid residues. This preprocessing ensured that the protein targets were appropriately prepared for subsequent molecular docking simulations with selected phytocompounds (Hameed *et al.,* 2020).

Preparation of Ligands

The 2D structure of ligands (alpha- spinasterol, ascorbic acid, chlorogenic acid, paracetamol, and diclofenac) were taken from PUBCHEM and drawn on Chemsketch. Their 3D structure was processed in PyMOL and exported in PDB format (Hameed *et al.,* 2020).

Amino acid Processing

Amino acids of proteins (PTGS2, TLR2, and TRPV4) were selected from CAST-P an online tool used for surface topography of proteins (Forouzanfar *et al.,* 2019).

Molecular Docking Mechanism

All selected ligands and proteins were docked with PYRX and an online tool used for molecular docking. A grid box has been made, and all amino acids were resided in that box. With protein PTGS2, ligands of plant *Citrullus colocynthis* that were alpha-sitosterol and standard drug diclofenac were docked, and its center were x= 46.4, y= 49.28, and z= 21.79 and size _x = 23.6, size _y= 14.53, and size _z= 24.88. With protein TLR2, ligands such as ascorbic acid and paracetamol (a standard drug) were docked, and their centers were x= 3.40, y= -27.4, and z= -16.02 whereas size x= 16.10, y= 28.18, and z= 20.13. With protein TRPV4, ligands such as chlorogenic acid and diclofenac (a standard drug) were docked, and its center x= 178.93, y= 130.69, and z= 118.38 and size x= 20.63, y= 15.25, and z= 18.15 (Banjo *et al.,* 2021)

Statistical analysis

Through the statistical results showing in the table on the following page, all activities on the seed part indicated the level of significance at $p<0.005$ by means of ANOVA. The molecular docking simulation predictions are as follows: catechin interacted with PTGS2, palmitic acid with TLR2, and *C. colocynthis* with TRPV4. Based on the docking simulations it was established that both catechin and palmitic acid have relatively high binding energy values within the active zones of target proteins (Stein *et al.,* 2024; Chaudhary *et al.,* 2023).

RESULTS

The analysis of anti- inflammatory, anti- pyretic and analgesic activities of methanolic seed extract of *Citrullus colocynthis* on experimental rats. The present study focused on evaluating the analgesic, anti-inflammatory, and antipyretic activities of methanolic seed extract of *Citrullus colocynthis* using validated *in vivo* models. Analgesic potential was assessed through the acetic acid-induced writhing test in albino rats, while anti-inflammatory activity was measured using the carrageenan-induced paw edema method. Antipyretic effects were evaluated using a yeast-induced pyrexia model. The table 1 showed the comparison of different dosages for the anti-inflammatory effect of *Citrullus colocynthis* seeds and traditional medicine. The results showed that it significantly inhibited inflammation, especially at lower doses of 50 mg/kg and 200 mg/kg, and the resulting seeds outperformed some conventional treatments. The findings of this study implicated the effects of the seed extract as a potent anti-inflammatory agent, which warrants investigation of therapeutic methods and optimal dosages for clinical uses.

Table 1. Percentage Inhibition of anti-inflammation of *Citrullus colocynthis* vs dose of Extract and Standard.

Groups	Treated dose (mg/kg)	Paw volume	%inhibition of anti-inflammatory
Control	50	1	50%
	100	0.5	0%
	200	0.5	0%
	400	1	50%
Standard	50	1	50%
	100	0.5	33%
	200	1	50%
	400	0.5	100%
Seed	50	0.6	100%
	100	1.1	50%
	200	0.5	100%
	400	1.5	50%

The table 2 compared the *Citrullus colocynthis* seed extract with its normal antipyretic activity. The results showed medium to high fire resistance; 100 mg/kg and 400 mg/kg extracts provided 86% and 85% inhibition of inflammation. Conventional treatment showed high inhibition at all doses measured; This makes the effect of drugs such as antipyretics different from that of fruit seeds.

Table 2. Percentage Inhibition of anti-pyretic of *Citrullus colocynthis* vs dose of Extract and Standard

Groups	Treated dose (mg/kg)	Pyrexia	%inhibition of anti-pyretic
Control	50	1.8	0%
	100	0.83	0%
	200	0.19	0%
	400	1	0%
Standard	50	1.05	85%
	100	0.59	37%
	200	3.58	75%
	400	2.82	76%
Seed	50	1.1	62%
	100	0.23	86%
	200	0.56	74%
	400	2	85%

The table 3 compared the analgesic effects of *Citrullus colocynthis* seeds compared to conventional treatments. Seed extracts were highly inhibitory; The maximum inhibition level (100%) was reached at a dose of 400 mg/kg, indicating analgesic properties. Treatment was generally dose dependent and in laboratory tests it can be as high as 85% at high doses.

Table 3. Percentage Inhibition of analgesic of *Citrullus colocynthis* vs dose of Extract and Standard

Groups	Treated dose (mg/kg)	writhing	%inhibition of analgesic
Control	50	7.48	0%
	100	8	0%
	200	7.45	0%
	400	7.5	0%
Standard	50	10.23	47%
	100	11.21	45%
	200	13.09	72%
	400	14.03	85%
Seed	50	12.08	63%
	100	13.22	65%
	200	14	88%
	400	17.04	100%

The results demonstrated the potential of methanolic extract as a unique medicine, with some doses having the same therapeutic effect. These findings encourage further research into the therapeutic potential and mechanisms of action of this agent, supporting its potential in drug development.

***In- Silico* Anti- inflammatory, Anti- pyretic and Analgesic activities**

Analysis of Protein Structure:

PTGS (prostaglandin-endoperoxide synthase 2) belongs to a very important enzymes family peroxidase. The protein consists of four chains (A–D), comprising a total of 604 amino acids and a molecular mass of approximately 69.1 kDa. Vadar analysis of PTGS2 showed the statistics percentage of alpha, coil and helix. Helix contains 39%, beta contains 13%, coil contains 47% and turn contains 23%. Ramachandran plot of PTGS2 protein showed that there are 96.13% outliers in preferred region, 3.50% in allowed region and 2 outliers remaining. TLR2 is a toll-like receptor protein. It consists of single chains A having 704 amino acids and molecular mass of 89.4kDa. Vadar analysis of TLR2 protein shows that 12% helix, 39% beta, 47% coil and 28% turn. Ramachandran plot of TLR2 protein showed that there are 91.80% outliers in preferred region, 6.94% in allowed region and there are 4 outliers remaining. TRPV4 consist of 4 chains from A to D with 871 amino acids and molecular mass of 98.01kDa. Vadar analysis of TRPV4 showed 60% alpha helix, 9% beta, 305 coil and 17% turn. Ramachandran plot of TRPV4 consist of 94.61% outliers in preferred region, 5.04% in allowed region and 2 outliers remaining, indicating structural representation in figure 1.

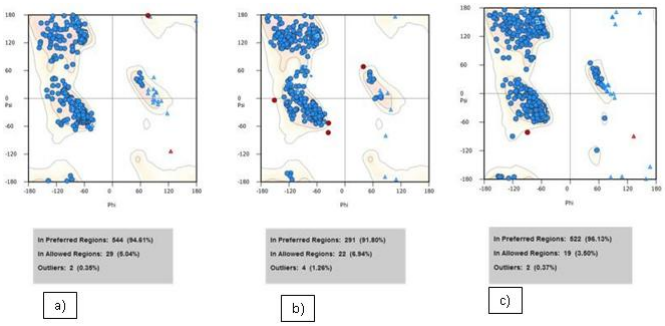


Figure 1: RAMACHANDRAN plot of a) TRPV4 b) TLR2 c) PTGS2

Analysis of Ligands structure

Alpha-spinosterol, a phytosterol with the chemical formula $C_{29}H_{48}O$ and a molecular weight of 412.7 g/mol, is associated with anti-inflammatory activity. Ascorbic acid, a water-soluble vitamin with the chemical formula $C_6H_8O_6$ and a molecular weight of 176.12 g/mol, exhibits both anti-inflammatory and analgesic activities. Chlorogenic acid ($C_{16}H_{18}O_9$; 354.31 g/mol) has also been reported to possess analgesic properties. Diclofenac ($C_{14}H_{11}Cl_2NO_2$; 296.15 g/mol) is a widely used standard pharmaceutical agent known for its potent anti-inflammatory and analgesic effects. Similarly, paracetamol ($C_8H_9NO_2$; 151.16 g/mol) is commonly employed as a reference drug for its antipyretic activity, indicating the structural representation in figure 2.

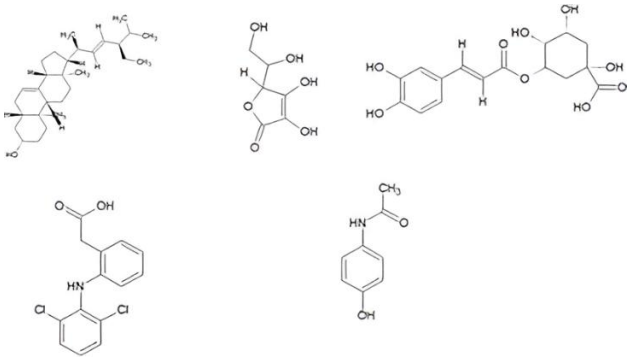
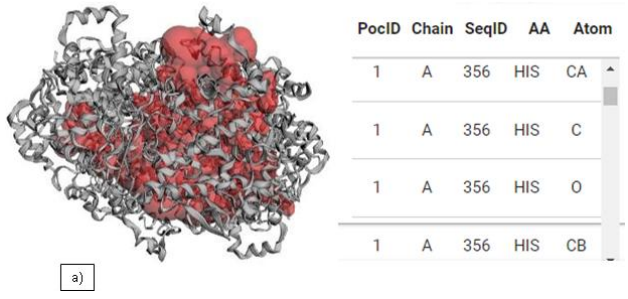
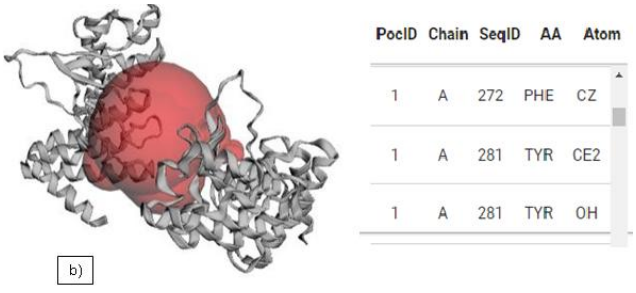


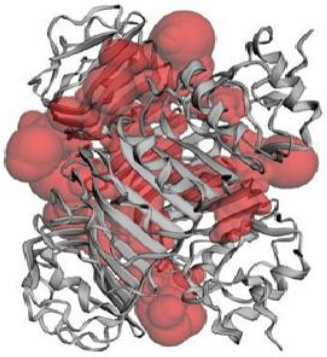
Figure 2: 2D structure of a) Alpha- spinosterol b) Ascorbic acid c) Chlorogenic acid d) Diclofenac and d) Paracetamol.

Prediction of Active site of PTGS2, TLR2, and TRPV4:

The active site of PTGS2 contains 12 amino acids as shown in figure 3a, b and c, indicating the ARG 311, ASP 314, ILE 315, GLN 318, GLU 319, HIS 320, PRO 321, GLU 322, TRP 323, GLY 324, GLU 326, GLN 327. The active site of TLR2 contains 10 amino acids LEU 280, GLU 281, GLU 283, SER 285, ARG 286, ASN 287, GLN 288, LEU 289, LYS 290, and SER 291. The active site of TRPV4 contains 11 amino acids LEU 350, LYS 353, ALA 354, PHE 357, PRO 358, SER 360, ASN 361, LEU 362, GLU 363, GLU 364, and ARG 392.







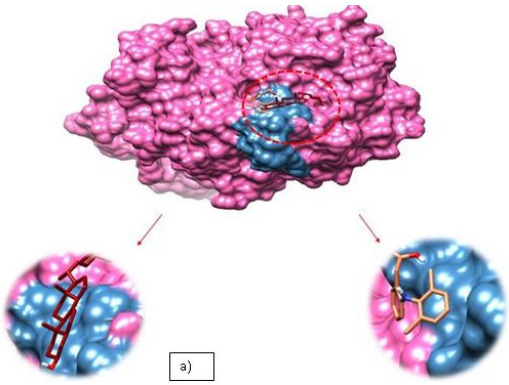
PocID	Chain	SeqID	AA	Atom
1	B	287	ASN	C
1	B	287	ASN	O
1	B	288	GLN	N

c)

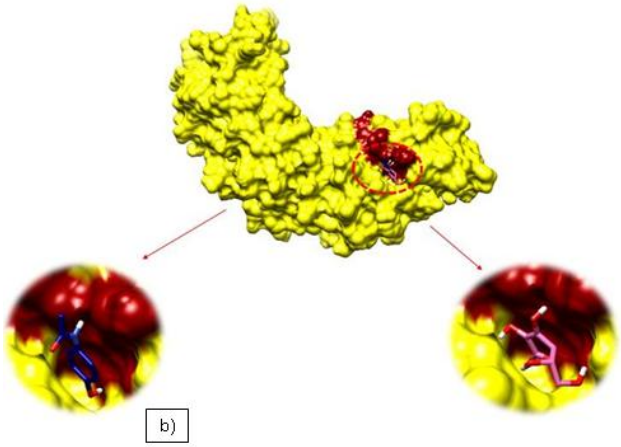
Figure 3 (A, B and C): shows binding pocket of PTGS2, TRPV4, TLR2 and its amino acids selected.

Interaction of ligands with proteins

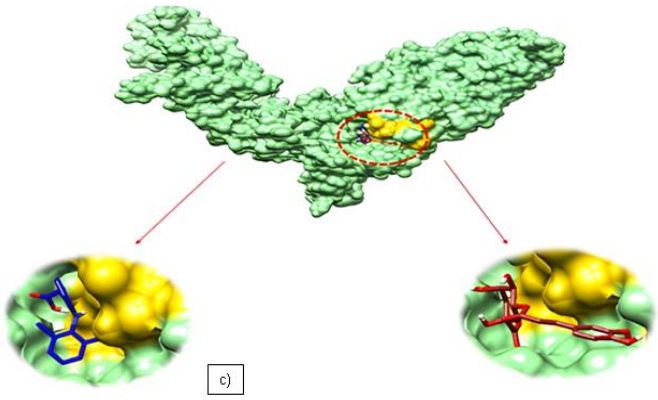
After molecular docking of selected ligands and proteins, all ligands residing at one place shows their binding interaction as shown in table 4 and figure 4a, b and c.



a)



b)



c)

Figure 4 (a): shows binding interaction of alpha- spinosterol and Diclofenac with PTGS2. **(b):** shows binding of ascorbic acid and paracetamol with TLR2 **(c):** shows binding interactions of chlorogenic acid and diclofenac with TRPV4.

The table 4 highlighted the amino acids found in the binding sites of various protein binding domains. The hydrogen bond is shown in red.

	Alpha- spinasterol	Ascorbic acid	Chlorogenic acid	Diclofenac	paracetamol
PTGS2	ARG 311, ASP 314, ILE 315, GLN 318, GLU 319, HIS 320, PRO 321, GLU 322, TRP 323, GLY 324, GLU 326, GLN 327			ARG 311, ASP 314, ILE 315, GLN 318, GLU 319, HIS 320, PRO 321, GLU 322, TRP 323, GLY 324, GLU 326, GLN 327	
TLR2		LEU 280, GLU 281, GLU 283, SER 285, ARG 286, ASN 287, GLN 288, LEU 289, LYS 290, SER 291			**LEU 280, GLU 281, GLU 283, SER 285, ARG 286, ASN 287, GLN 288, LEU 289, LYS 290, SER 291**
TRPV4			**LEU 350, LYS 353, ALA 354, PHE 357, PRO 358, SER 360, ASN 361, LEU 362, GLU 363, GLU 364, ARG 392**	**LEU 350, LYS 353, ALA 354, PHE 357, PRO 358, SER 360, ASN 361, LEU 362, GLU 363, GLU 364, ARG 392**	

For example, PTGS2, also called COX-2, showed significant interactions with various ligands between ARG 311 and ASP 314, while hydrogen bonding was observed only with chlorogenic acid and paracetamol. TLR2 has shown several amino acid interactions; Hydrogen bonding was observed with ascorbic acid. TRPV4 maintains stable interactions at residues such as LEU 350 and GLU 363 to mark single ligand binding sites. These results provided insight into specific molecular mechanisms that play an important role in understanding the dynamics of ligand-protein interactions and their therapeutics potential role in those mechanisms.

Docking with PTGS2, TLR2, and TRPV4

Docking of PTGS2 with selected ligands (alpha-spinosterol and diclofenac) showed that alpha- spinosterol showed hydrogen bonding with ARG 311 and ASP 314 with the bond distance of 2.02A and 2.28A. Diclofenac showed hydrogen bonding with ARG 311 and ILE 315 with the bond distance of 2.02 A and 2.28 A. Docking of TLR2 with Ascorbic acid and Paracetamol showed that ascorbic acid showed hydrogen bonding of SER 291 with bond distance of 2.31 A and Paracetamol showed hydrogen bonding of ARG 286 with bond distance 2.43 A. While docking with TRPV4 of chlorogenic acid and diclofenac showed that chlorogenic acid made hydrogen bonding of ASN 361 and ARG 392 with bond distance of 2.47 A and 2.11 A, whereas diclofenac showed hydrogen bonding of GLU 363 and ARG 392 with bond distance of 1.74 A and 2.11 A as shown in table 5 and figure 5a, b and c.

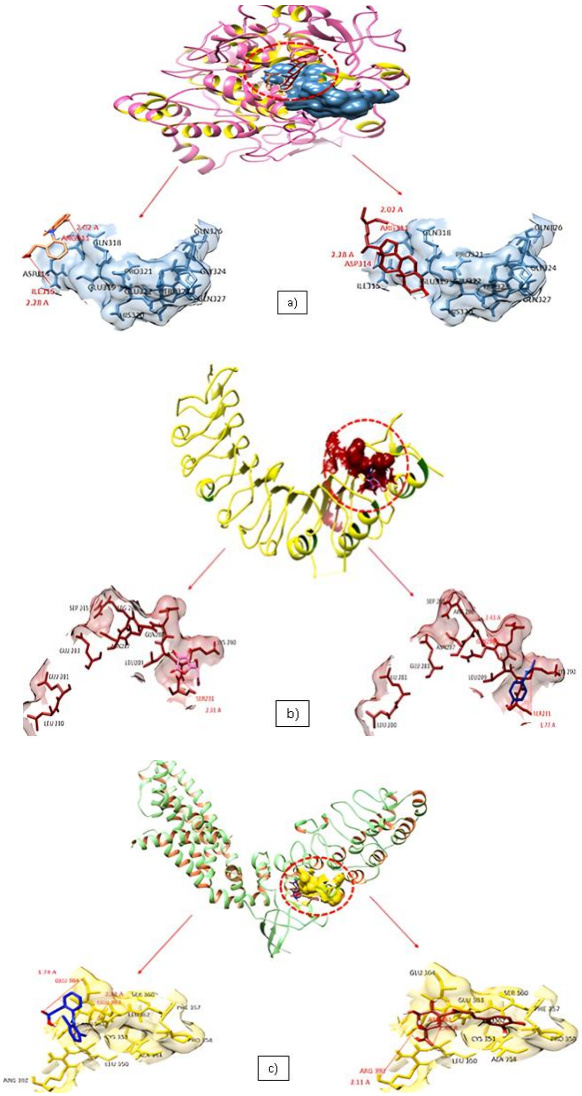


Figure 5 (A): shows docking process of PTGS2 with alpha- spinosterol and diclofenac. (B) shows docking process of TLR2 with ascorbic acid and paracetamol (C) shows docking process of TRPV4 with chlorogenic acid and diclofenac.

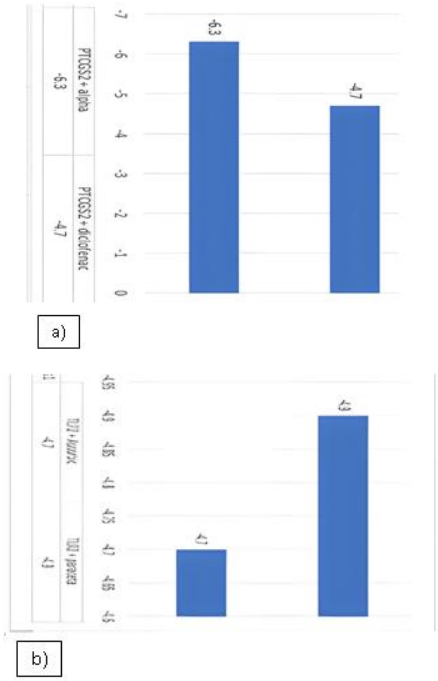
Table 5 highlighted the hydrogen bonding and distance between different ligands and proteins. In the case of alpha-spinosterol and diclofenac against PTGS2, the results showed hydrogen bonding between ARG 311 and ASP 314 at distances of 2.02 Å and 2.28 Å. In the case of the ascorbic acid antagonist TLR2, the interaction was mediated by hydrogen bonding of SER 291 at position 2. 31Å. Chlorogenic acid binds to ASN 361 and ARG 392 in TRPV4 at distances of 2.47 Å and 2.11 Å, respectively. Paracetamol interacts with ARG 286 in TLR2 channel 2. 43 A. With diclofenac, hydrogen bonds form with GLU 363 and ARG 392 of TRPV4 at distances of 1.74 Å and 2.11 Å. All these interactions define the key residues and interactions required for binding and hence the bioactivity of the ligands.

Table 5: Shows hydrogen bonding and bond distance of ligands with proteins

Ligands	Hydrogen bonding	Bond distance	Protein
Alpha-spinosterol	ARIGININE 311 ASPARTIC ACID 314	2.02A 2.28A	**PTGS2**
Ascorbic acid	SERINE 291	2.31 A	**TLR2**
Chlorogenic acid	ASN 361 ARG 392	2.47 A 2.11 A	**TRPV4**
Diclofenac	ARIGININE 311 ISOLEUCINE 315	2.02 A 2.28 A	**PTGS2**
Paracetamol	ARIGININE 286	2.43 A	**TLR2**
Diclofenac	**GLU 363 and ARG 392**	**1.74 A 2.11 A**	**TRPV4**

Binding energy

Binding of PTGS2 with alpha-spinosterol and diclofenac, TLR2 with Ascorbic acid and paracetamol, and binding of TRPV4 with Chlorogenic acid and Diclofenac was done using PYRX as shown in figure 6a, b and c. Highest binding energy of alpha spinosterol and diclofenac with PTGS2 were -6.3 and -4.7 whereas highest binding energy of ascorbic acid and paracetamol with TLR2 were -4.7 and -4.9. Highest binding energy of Chlorogenic acid and diclofenac with TRPV4 were -6.5 and -6.3.



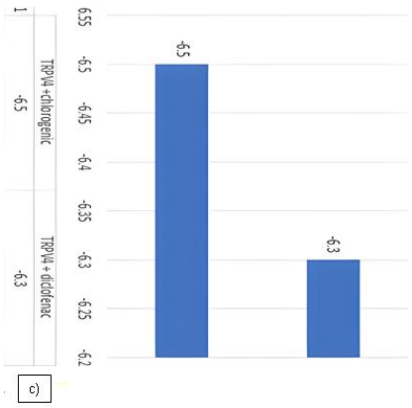


Figure 6 (a, b &c): shows hydrogen bonding of ligands when docked with proteins.

DISCUSSION

This study investigates the pharmaceutical potential of *Citrullus colocynthis* methanolic extract of seeds, focusing on its anti-inflammatory, antipyretic and analgesic properties of phytoconstituents. *In silico* analysis by application of various advanced bioinformatics tools performed to confirm its best therapeutic potential at molecular level. The *Citrullus colocynthis* commonly referred to as bitter melon or colocynth. *Citrullus colocynthis*, commonly known as bitter apple or colocynth, is a medicinal plant native to regions extending from the Mediterranean Basin to North Africa and the Middle East (Abid *et al.,* 2025; Kiran *et al.,* 2025; Zahra *et al.,* 2025; Afsar *et al.,* 2024; Aziz *et al.,* 2024; Ejaz *et al.,* 2024; Farah *et al.,* 2024; Li *et al.,* 2022; Caesar *et al.,* 2022; Behera *et al.,* 2021). However, the plant is toxic, yet different parts of the plant have been in earlier times employed in various medicinal systems for the treatment of digestive ailments, skin diseases, and fevers. Care should be taken especially with production from old fruits because the introduction of toxic substances in large quantities is dangerous. Apart from petrochemical uses, other researchers have also investigated the phytochemical properties of plants that have active ingredients that have health benefits such as anti-inflammatory and antioxidant effects (Stein *et al.,* 2024). Although bitter melon is widely shunned for this reason, bitter melon has been brought into the fold of food in societies of the world. In Asian meals, this plant is often used in recipes, bitterness is always blended with other tastes. They have also been widely employed in culinary confectionaries as flavouring agents that bring out the bitterness and contribute taste to the dishes and beverages for the daily household purposes of uses in the domestics (Chaudhary *et al.,* 2023).

A great number of medicines prepared with the help of bitter apples are well-established in traditional systems of medicine in different parts of the world. They were also employed to facilitate digestion, purgatives, and medications for some diseases that apply the old Indian system of Ayurveda, and China's Traditional Medicine systems. Harsh fruits were considered to regulate the tumors of the body and purify and heal the body. Specifically, the migration of bitter apples to different continents was made possible by early explorations and trade ways. Such fruits were transported throughout the Silk Road, Mediterranean, and maritime routes, and later, these products were the most sought-after instruments used for pharmaceutical purposes as well as culinary to add value to the cultures and economy of any country and its importance in the economic situation (Yusuf *et al.,* 2023). The contemporary scientific analysis of the literature suggests that bitter apples contain several chemical compounds and hold some level of therapeutic health qualities. Flavonoids, alkaloids, and terpenoids have been subjected to research about their antioxidant, anti-inflammation, and anti-diabetic characteristics (Khalid *et al.,* 2025; Benkiran *et al.,* 2024)). Small, yellow, often fragrant, flowers form a raceme touching the ground and are mostly unisexual. Many are round, some resembling small melons or gourds with a sponge like pulpy substance that is bitter. Root structure that provides food and water to the plant and adapted this area for dwelling in the desert. Palmate cleft with margin sinuates-serrated and the leaves are arranged in an opposite or sub-opposite manner along the stem. Its extracts may decrease inflammation by suppressing the production of pro-inflammatory mediators, acting as a source of antioxidants, affecting the immune system, interacting with enzymes, and by affecting intracellular signalling (Singh *et al.,* 2023).

Another of the bitter apple traditional uses is as an antipyretic to cure fevers. This may partly explain the antipyretic action that may be attributable to its anti-inflammatory actions, alteration of immune response, and possible antimicrobial properties. The relative toxicity makes it important to go through clinical trials and get scientific validation. Bitter apple extracts lower the pain potentially due to their anti-inflammatory effects, alteration of transmitters, antioxidant activity, and suppression of pain-inducing enzymes. Some of the edited and reviewed studies and research that are included in the document are the Pharmacological activities of *Citrullus colocynthis*. Some scientific studies confirmed the plant's ability to help combat inflammation, fever, and pain in Phytopigments. In a related study conducted by Singh and his colleagues (Singh *et al.,* 2023) on *Cucumis melo* subsp. agrestis, the hydro-alcoholic extracts of both fruits and leaves exhibited significant anti-inflammatory and wound healing properties. In the carrageenan-induced rat paw edema model, the fruit extract showed 67.85% inhibition of inflammation ($p < 0.05$), while the leaf extract resulted in 75% inhibition ($p < 0.01$). Similarly, in the chronic cotton pellet granuloma model, the inhibition of granuloma formation was 68% with the fruit extract ($p < 0.05$) and 74% with the leaf extract ($p < 0.01$). Moreover, in a burn wound healing model, the percentage of wound contraction was recorded at 70.2% for the fruit extract and 75.3% for the leaf extract, indicating a significant improvement in wound closure over time (Singh *et al.,* 2023; Zahra *et al.,* 2023).

The present study on *Citrullus colocynthis* demonstrated strong anti-inflammatory, antipyretic, and analgesic activities, particularly at higher doses (200–400 mg/kg), with inhibition reaching up to 100% in analgesic models that showed consistency with the previous study conducted on *Citrus paradisi* leaf extract, that showed high anti-inflammatory (80%) and antipyretic (90%) effects but relatively lower analgesic inhibition (36%). Both studies used similar *in vivo* models and standard drugs for comparison. *In silico* results from each study supported the biological findings, with key phytochemicals showing strong binding to PTGS2, TRPV4, and TLR2. Overall, *Citrullus colocynthis* exhibited broader and more potent pharmacological activity, especially in analgesic models [30]. Other works also indicate the possibility of therapeutic use of the chemical substances contained in the plant matter, for example, catechin and palmitic acid which may exhibit considerable therapeutic effects. *Citrullus colocynthis* is a plant that has been used since the plant was present in the Mediterranean region and has been used for ages in traditional medicine besides cooking (Zahra *et al.,* 2023; Ganesh *et al.,* 2021; Pradhan *et al.,* 2021). It's still being investigated by modern research for its probable pharmacological effects, especially antirheumatic, antipyretic, and analgesic roles. Nevertheless, owing to its lethal effect on the organism, further research is required to elucidate the effects of pyrolusite and its usage to treat pathological states. Before using them for treatment, it is highly advised to consult with healthcare professionals (Fuller *et al.,* 2024; Douglass *et al.,*2020). Summarizing this conversation, it is possible to state that *Citrullus colocynthis* has been at the center of many cultures playing various roles as food, medicine, and commodity and at the same time dangerous as a poisonous plant that could even lead to death; it is evidence of a long-standing symbiotic relationship between the plant and that in humans Douglass *et al.,*2020

CONCLUSION

In the present study, we investigated about pharmacological effects with emphasis on anti-inflammatory, anti-pyretic and analgesic effect of methanolic seed extract of *Citrullus colocynthis.* It pointed out the pharmacological effects of *Citrullus colocynthis*, with special attention to the impact made on anti-inflammatory, analgesic, and antipyretic effects. The outcome of the experiments clearly showed that the extracts of this plant considerably affected inflammation and pain in albino rats. The docking studies revealed that the active compounds of the plant were found to have good binding interactions with the target proteins, hence good evidence of the plant's active therapeutic effects. Therefore, the study demonstrates that *Citrullus colocynthis* should be valued as a prospective bio-agent in both medicinal field and future research open new horizons of its applications in agricultural fields. It is safe for the environment since it is naturally decomposable and hence can be used in pest control thus promoting eco-friendly farming. Therefore, this study calls for the continued research and use of *Citrullus colocynthis* and other medicinal plants as sources for other natural cures for different ailments and a solution to crop-related issues. Harnessing the therapeutic potential of medicinal plants like *Citrullus colocynthis* offers promising avenues for addressing widespread health conditions while also contributing to environmentally sustainable practices.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. No conflict of interest. All the authors declare no conflict of interest.

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