In-Vivo AND *In-Silico* ANALYSIS OF ANTI- INFLAMMATORY, ANALGESIC, AND ANTI PYRETIC ACTIVITIES OF *Citrus paradisi* LEAF EXTRACT

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ABSTRACT

Medicinal plants recently gained attention due to the presence of many phytochemicals involved in various therapeutical activities. The main aim of this study was to determine the *in-vivo* and *in-silico* anti-inflammatory, analgesic, and anti-pyretic effects of *Citrus paradisi* leaf ethanolic extract using albino rats (n = 36). For inducing inflammation, pain, and fever in albino rat's carrageenan, acetic acid dilution in distilled water and yeast dilution in saline were used. The four different concentrations (50, 100, 200, and 400mg/kg) of ethanolic extract of *Citrus paradisi* leaf were used to prevent inflammation, pain, and fever. Diclofenac and paracetamol were used as standard drugs in this study. The ethanolic extract of *Citrus paradisi* leaf showed efficient anti-pyretic and anti-inflammatory inhibition (90% and 80%, respectively) but less efficient analgesic inhibition (36%). Similarly, *in-silico* study was done using leaf bioactive compounds such as linalool, beta-pinene, geraniol, citral, and terpinene-4-ol as ligand molecules and proteins for anti-inflammatory, analgesic, and anti-pyretic activity were PTGS2, TRPV4, and TLR2, respectively. The process of docking was done using ligand and protein molecules. The results of *in-silico* study were the same as *in-vivo* study; the binding energy values of anti-inflammatory and anti-pyretic activity were more efficient than an analgesic. In summary, the ethanolic extract of *Citrus paradisi* leaf in *in-silico* and *in-vivo* studies proved less efficient against pain while more efficient against inflammation and fever.

Keywords: TLR2, TRPV4, PTGS2, *Citrus paradisi*, Molecular docking (MD), Inflammation, Pain and Fever.

INTRODUCTION

From ancient times, herbal medicine obtained from the Vedic era has been used for treating many disorders, including heart diseases, cancer, digestive system disease, central nervous system (CNS) diseases, and diabetes (1-5). Various placebo systems like Ayurvedic, Asians, Chinese, and Unani widely use the therapeutic property of plants as a medicament (6). It diverts the attention of people from pharma medication to ayurvedic medicine. Even for validating the therapeutic herbal products, WHO (7) has suggested a specific procedure using advanced technologies and conventional approaches (8-13). In Asia, 250,000 to 500,000 plant species out of 4.5 million have been examined for many biological activities that, include the Rutaceae family.

One of the most significant members of the citrus genus is *Citrus paradisi Macfad*. It belongs to the family Rutaceae, found all over the world. Its common name is bitter fruit (due to its bitter taste) and grapefruit (due to cluster like grapes). It is mainly cultivated in Barbados Island, but also in Morocco, Israel, Brazil, Jamaica, Asia, Mexico, USA, Florida, California and Spain etc. Now, in Pakistan, grapefruit, due to its medicinal and many other uses (essential oils, perfume, cosmetics and detergents) become the most significant crop and fruit (14-15). The *C. paradisi* contains tannins, terpenoids, kaempferol, alkaloids, phenols, basic oil, saponins, steroids, tri-terpenoid, and flavonoids. The *C. paradisi* leaves to some extent are also essential for many pharmacological activities including antioxidant, anti-inflammatory, analgesic, antitumor, antipyretic, cytotoxic, and antimicrobial (15)). Leaves are also effective against swelling, insomnia, ulcers, gout, cuts and wounds, arthritis, and infections (8). Similarly, grapefruit leaf essential oils also show anti-cancerous, antioxidant and, anti-pyretic activity due to different phytochemicals such as linalool, terpinene-4-ol, Beta-pinene, limonene, p-cymene, and alpha-pinene (16-17). Grapefruit contains a very strong component lycopene that plays a significant role in decreasing the development of prostatic cancer and beta-carotenes against gastrointestinal tract (GIT) cancer (18).

Medicinal plants due to the presence of different bioactive compounds, play an important role in drug discovery development. In order to overcome conventional drugs' side effects, natural drug development is on the rise (3-4) . Therefore, different computational approaches gain more attention due to the availability of different online databases (19). Inflammation normally provides the body's defense against many infections. However, if the inflammation becomes long-lasting and cannot be cured at the right time, then it has negative consequences on the body. Therefore, many plant phytochemicals were studied for their anti-inflammatory properties (10-13). The target proteins for the inflammation process can be used, including PTGS2, COX-1, COX-2 (20). Prostaglandin is the lipid derivative that maintains the body's homeostatic function and mediates the inflammatory response. They produce from arachidonic acid by the action of cyclooxygenase. Their production becomes high in the inflamed tissues and may function both in the induction and inhibition of inflammation (21).

Pain results from a protective response against harmful stimuli in organ dysfunction or its function imbalance. Several drugs are used against pain, such as aspirin, morphine, and diclofenac etc., but due to their side effect, the natural drugs gain attention. *C. paradisi* leaf contains some phytochemicals, including alpha and beta-pinene, geraniol, p-cymene, and citronellol (14) for analgesic activity (20). Trpv4 can be used as a target protein against target ligand molecules for analgesic activity (21-22). The TRPV family is mainly linked with CNS, and functions include memory, pain, and mechanical sensation. It contains six subtypes such as TRPV1 to TRPV6, and TRPV4 is mainly linked with mechanical sensation and many kinds of pain medications such as pain resulting from AIDS therapy, mechanical hyperalgesia, diabetes, and vincristine chemotherapy (23).

Pyrexia is induced in the body as the symptom of various diseases or due to pathogens entry. Untreated pyrexia can cause serious health consequences. Several drugs including paracetamol and aspirin are used to treat pyrexia; however, due to their side effects, natural drugs can be preferred against pyrexia. *C. paradisi* leaf contains some phytoconstituents including terpinene-4-ol, alpha-terpinene, alpha-pinene, beta-pinene and citral (14) that can be used as a therapeutical agent against fever (24). TLR2 protein can be used as a target protein against therapeutical agents in antipyretic activity. It performs the transmembrane signaling receptor activity. It produces mainly inflammatory and innate immunity responses. The objective of this study was to evaluate the analysis of anti- Inflammatory, analgesic, and anti-pyretic activities of *Citrus paradisi* leaf ethanolic extract both *in-Vivo* and *in-Silico*. TLR2 activates the endotoxic protein, which further produces IL-6 and TNF-alpha that induce fever in the body of rats (25).

MATERIALS AND METHODS

Sampling

Fully developed leaves of *Citrus paradisi* were collected from the field region of Sahiwal, Punjab and botanical garden of the University of Punjab Lahore, Pakistan. After collection, leaves were cleaned and dehydrated at 37ºC. For investigating the anti-inflammatory, analgesic, and antipyretic activity, albino

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rats (n = 36) of the female sex (140-170g) were studied. All the rats were brought from the animal house of the University of Lahore. The rats were kept in polypropylene cages at fasting, and then distilled water was given to them before the experiment. Ethical approval was obtained from the Bioethical, Biosafety, and Biosecurity Committee, University of Lahore.

Extract Preparation

Extract of Leaves

The leaves of *C. paradisi* were dipped in ethanol in a sterile beaker or glass bottle for making mixtures. After that, the mixture of leaves and ethanol was shaken well for 5 minutes and then left for 10-15 days at room temperature. To filter the mixture, standard size whatman filter paper was used on the 16th day, and after pouring it into a petri-dish, the mixture was left at 37ºC for one week in an incubator. After drying, the mixture was poured into eppendorf and labeled with a permanent marker.

Drugs and Chemicals

1. Distilled water 2. *Citrus paradisi* leaf extract (400mg/kg) 3. Diclofenac (100mg/kg) from University of Lahore Teaching Hospital 4. Paracetamol (400mg/kg) from University of Lahore Teaching Hospital 5. Yeast-induced pyrexia (400mg/kg) 6. Acetic acid induced pain (400mg/kg) 7. Normal saline (400mg/kg).

Anti-inflammatory Activity Model

Inflammation in the albino rats' paw was caused by the carrageenan and reduced by the diclofenac and ethanolic leaf extracts of *C. paradisi* (50, 100,200 and 400mg/kg). A total of 36 albino rats were equally divided into three groups (control, standard, experimental). In the control group, rats were treated with normal saline. In the standard group, rats were treated with diclofenac (100mg/kg), and in the experimental group, rats were treated with the *C. paradisi* leaf aqueous extract. First, size of the hind paw of rats was measured, followed by injecting the carrageenan doses (50, 100, 200, and 400mg/kg) into the hind paw at sub-planter region. The carrageenan doses start producing inflammation in the rat's hind paws. After 3 hours of carrageenan injection, the doses of *C. paradisi* leaf extract and diclofenac (12.5, 25, 50, and 100mg/kg) were injected into the hind paw, and the paw size was measured at the time interval of 1, 2, and 3hr.

Analgesic Activity Model

In this activity, abdominal pain or muscle contraction in albino rats was produced. Pain was produced by acetic acid and then recovered by leaf extracts of *C. paradisi* (50, 100,200 and 400mg/kg). The rats were divided in this model according to the anti-inflammatory activity model. By injecting acetic acid, the writhing process occurred in rats. It was injected to determine the potential of leaf ethanolic extracts of *C. paradisi* in pain process. In experimental and standard groups, acetic acid 5(0, 100, 200 and 400mg/kg of body weight) were injected. But before 1 hour of the experiment, the doses of *C. paradisi* leaf ethanolic extract and diclofenac doses of 12.5, 25, 50 and 100mg/kg were injected into rats intraperitoneally. The rats were put into different cages during the activity and the complete activity done within 20 to 25 minutes.

Anti-pyretic Activity Model

Pyrexia is induced in antipyretic activity in albino rats by normal saline and yeast and then treated with leaf ethanolic extracts of *C. paradisi* (50, 100,200 and 400mg/kg) for reducing fever. The rats in this model were also divided according to the anti-inflammatory activity model. In control group, rats were treated with normal saline (2ml/kg) below the nape of the neck, and in the standard group, rats were treated with normal saline + brewer's yeast. In the experimental group, rats were treated with doses of ethanolic leaf extracts of *C. paradisi*. After injecting yeast, fever developed after 21 hours, and the highest temperature, 101.81 Fahrenheit was measured.

Statistical analysis

ANOVA was used to analyze the result of the activities and efficient difference ($P<0.005$) was accepted in all activities. The mean ±S.D was used to present the results of experiment. The student test for unpaired comparison of other group activities was countered for statistical importance of difference. While the difference ($P<0.005$) was considered to be efficient.

Computational Methodology

Different tools of bioinformatics, including Chemsketch, Chimera 1.15, PyMOL, PyRx, and Discovery studio were used for the evaluation of *in-silico* anti-inflammatory, analgesic, and antipyretic activity of *C. paradisi* leaf extract. Macromolecules can be visualized in three dimensions using PyMOL, a cross-platform molecular graphic tool. Numerous plugins, such as macromolecular analysis, homology modelling, protein-ligand docking, pharmacophore modelling, VS, and MD simulations, have significantly improved PyMOL's functionalities. Molecular structures and associated data, including density maps, trajectories, and sequence alignments, can be interactively visualized and analyzed using the tool Chimera. PyRx is a program for virtual screening that can be used in computational drug discovery to check libraries of compounds against possible therapeutic targets. Pharmaceutical Chemists can execute virtual screening using PyRx from any platform. The software supports users at every stage of the procedure, from data preparation to job submission and outcome analysis. PyRx is a useful tool for computer-aided drug design since it has a docking wizard and an intuitive user interface. The inflammatory protein PTGS2, analgesic protein TRPV4, and antipyretic protein TLR2 were selected and retrieved from SWISS-PROT in the form of PDB, but if protein structure was not available at PDB, then the homology modeling approach was used. For observing the hydrophobic interactions and 3D structure of target proteins and ligands, the Discovery Studio R2 Client was used. The PDB structure of target protein was used to access the Ramachandran graph (26).

Protein Preparation

For protein preparation, the 3D structure of all three proteins such as PTGS2, TLR2, and TRPV4 was taken from Swiss Prot (https://swissmodel.expasy.org/interactive) with sequence entries P35355, Q6YGU2, and Q9ERZ8, respectively and then opened in Chimera and selected only one chain of all three proteins. The protein structures can also be retrieved from other databases such as UniProtKB/ Protein Data Bank (www.rcsb.org). All other molecules like water and metals were removed before the protein processing. Geometry of all hetero groups was checked, hydrogen atoms were added if required, and then the structure of protein in protein data bank was saved in pdb format.

Ligand Selection

The most abundant and specifically responsible ligands for each activity were selected. Two ligands of *C. paradisi* and one standard drug for each activity were selected for each protein. Selected ligands for specific activity are given below in Table 1.

Table 1: Bioactive compounds of *C. paradisi* leaf as ligand molecule for analgesic, anti-inflammatory, and anti-pyretic proteins.

Compounds Name	Biological activity	Molecular formula	PubChem ID
Linalool	Anti-inflammatory	$C_{10}H_{18}O$	6549
Beta-pinene	Anti-inflammatory and analgesic	C10H16	14896
Diclofenac	Anti-inflammatory and analgesic	$C_{14}H_{11}Cl_2NO_2$	3033
Geraniol	Analgesic	$C_{10}H_{18}O$	637566
Citral	Anti-pyretic	$C_{10}H_{16}O$	638011
Terpinene-4-ol	Ant-pyretic	$C_{10}H_{18}O$	11230
Paracetamol	Anti-pyretic	$C_8H_9NO_2$	1983

Ligand Preparation

The ligand molecules were *C. paradisi* leaf phytochemicals involved in different biological activities such as anti-inflammatory, analgesic, and antipyretic. For these activities, leaf bioactive compounds were extracted from literature or phytochemical database such as Dr. duke database (27). After that, we selected two targeting ligand molecules of *Citrus paradisi* plant for each activity. The 3D structure of ligand molecules was taken from the PubChem website (https://phytochem.nal.usda.gov/phytochem/plants/show/461?et=#act-14303-close) or otherwise, the 3D structure of ligand molecules was drawn into Chemsketch.

Selection of Binding Sites

The binding pocket for the ligand molecules was selected using online server depth residue (http://cospi.iiserpune.ac.in/depth). Atom/residue depth quantifies how far an atom/residue is buried within a bulk solvent. This straightforward measurement has a wide range of applications in describing the chemical and physical characteristics of protein structures. It correlates well with protein activity, 3D structural model correctness, hydrophobicity, structural stability, sizes of globular domains, and residue conservation. Furthermore, residue depth has been utilized to forecast the locations of protein-protein interaction hot spots, folding nucleation sites, phosphorylation sites, and small molecule-binding sites. In this process, the protein data bank (pdb) file of the required protein was uploaded and run on the server for predicting binding site amino acids. The results were presented in the form of graph of binding site residues and 3D structure of protein. The binding site default parameters were Blast maximum sequence of 1000, solvent neighborhood radius (4.2Å), and threshold probability (0.8).

Molecular Docking

The ligand molecules were selected to analyze the binding configuration of ligands with the target protein. Using the docking tool PyRx (VINA Wizard), the process of molecular docking on selected proteins and ligand molecules was performed. All the ligand molecules were *C. paradisi* leaf phytochemicals against anti-inflammatory, analgesic, and antipyretic protein such as PTGS2, TRPV4, and TLR2, respectively. The chemical structure of all ligands was retrieved from PubChem or sketched in the ChemSketch tool. For the energy minimization of each ligand separately, the UCSF Chimera tool with the parameters upgrade interval set at 10, steepest descent, and conjugate gradient steps 100 with the step size 0.02Å was applied.

In order to obtain efficient structure conformation of ligands, Gasteiger charges were added using Dock Prep. Finally, using the screening tool PyRx (20), the molecular docking assay was run on all the ligands against TRPV4, PTGS2, and TLR2 proteins. For better binding conformation analysis, the grid box for PTGS2 set in (X=25.23, Y=42.16 and Z=16.60), TLR2 set in (X=-2.33, Y=35.09 and Z=-19.84), and TRPV4 set in (X=138.37, Y=118.30 and Z=117.03) with default exhaustiveness value = 8 was performed. For moving ligand molecules freely in search space, enough grid box size was adjusted on binding site residues for good ligand conformation. Each ligand docks with the respective target protein separately, and then all docked complexes were observed closely for the significant docking results. All the docked complexes were analyzed on behalf of the binding pattern and binding affinity values (Kcal/mol) of ligand-protein complexes. The UCSF Chimera and Discovery Studio R2 Client were used for the graphical study of all docked complexes (26).

RESULTS

Paw edema induced by Carrageenan

In anti-inflammatory activity, the highest % inhibition shown by *C. paradisi* ethanolic leaf extract at the dose of 200mg/kg was 80% relative to control and standard group. The *C. paradisi* leaf extract at the doses of 50, 100, and 400mg/kg showed 14%, 36%, and 47% inhibition, respectively. In standard group, e rats treated with diclofenac and showed the highest inhibition at the dose of 400mg/kg that was 100% as compared to other doses 50, 100, and 200mg/kg with the percentage inhibition of 37.5%, 28.5% and 40% respectively (Figure 1). The maximum % inhibition was shown by experimental group (leaf ethanolic extract of *C. paradisi;* 80%) at the dose of 200mg/kg (Table 2; Figure 1).

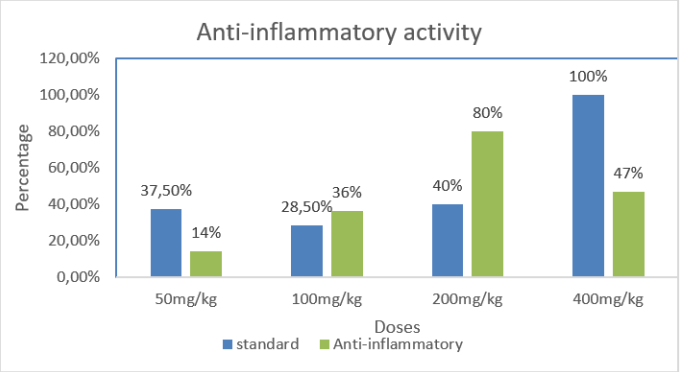

Figure 1: shows the comparison of % inhibition of diclofenac and *C. paradisi* leaf extract in anti-inflammatory activity.

Analgesic activity

In analgesic activity, the leaf extract of *C. paradisi* showed less efficient result as compared to standard group at 50, 100, 200, and 400mg/kg. The percentage inhibition (15%, 18%, 20% and 36%) of experimental group that were treated with ethanolic leaf extract of *Citrus paradisi* was shown at 50, 100, 200, and 400mg/kg, respectively. In the standard group, rats treated with the diclofenac (50, 100, 200, and 400mg/kg) showed the percentage inhibition 45.5%, 45%, 72% and 82%, respectively relative to experimental group (Table 2; Figure 2).

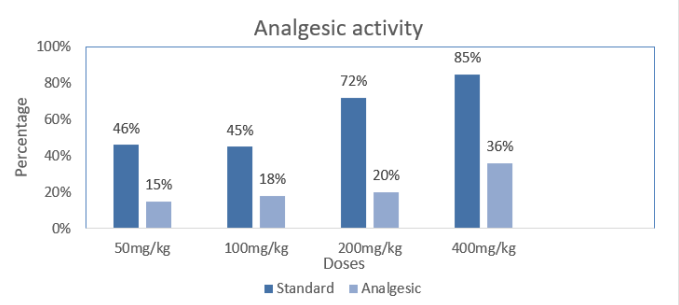

Figure 2: shows the comparison of % inhibition of diclofenac and *C. paradisi* leaf extract in analgesic activity.

Antipyretic activity

In antipyretic activity, the ethanolic leaf extract of *C. paradisi* showed efficient results at 50, 100, 200, and 400mg/kg doses. The highest % inhibition (90%) of antipyretic activity was shown at 400mg/kg of *C. paradisi* leaf ethanolic extract compared to the standard group. Significant dose-independent inhibition was seen in the standard group. Standard group was observed with maximum (85%) inhibition at 50mg/kg dose (Table 2; Figure 3).

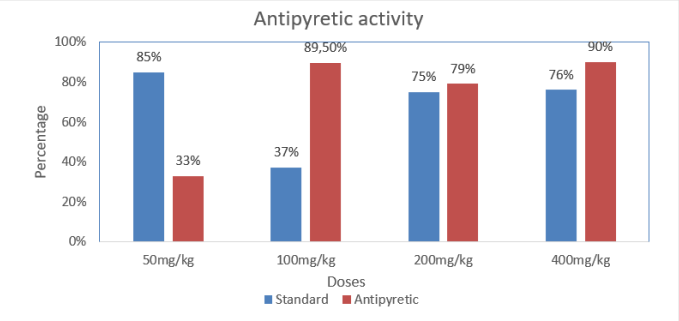

Figure 3: shows the comparison of % inhibition of paracetamol and *C. paradisi* leaf extract in antipyretic activity.

Table 2: The % inhibition of anti-inflammatory, analgesic, and antipyretic activities of control, standard, and experimental group. Experimental group: *Citrus paradisi* leaf ethanolic extract.

Groups	Treated dosew	% Inhibition of anti-inflammatory activity	% Inhibition of analgesic activity	% Inhibition of antipyretic activity
Control	50	0	0	0
	100	0	0	0
	200	0	0	0
	400	0	0	0
Standard	50	37.5	45.5	85
	100	28.5	45	37
	200	40	72	75
	400	100	85	76
Experimental group	50	14	15	33
	100	36	18	89.5
	200	80	20	79
	400	47	36	90

In-Silico Anti-inflammatory Activity

Inflammatory protein PTGS2

The protein PTGS2 (prostaglandin G/H synthase and cyclooxygenase), also called COX-2 or cyclooxygenase 2, plays an important role in renal injuries, tumor growth, and inflammation process but is strongly triggered in the course of inflammation. In the formation of prostaglandins, the COX2 enzyme catalyzes the rate-limiting step. The prostaglandins derived from arachidonic acid act as signaling molecules in the body. This intracellular PTGS2 expression and its resulting prostaglandin development facilitate the inflammatory cascade as well as overcome it gradually. Previously, mouse studies have shown important PTGS2 function in normal physiology and inflammation (28-29). The structure of PTGS2 is shown in Figure 4.

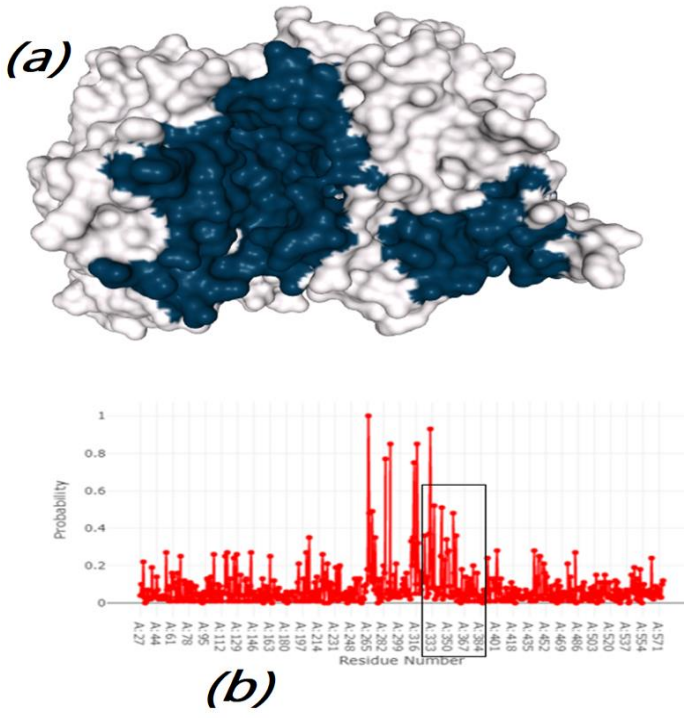

Figure 4 (a): Show PTGS2 protein chain A with binding pocket obtained from online server Depth residue. The highlighted amino acid residue in **(b)** such as given number 333-384 of PTGS2 protein show high probability of binding with ligand molecules such as beta-pinene and linalool.

Binding pocket selection

The binding site of PTGS2 protein for ligand molecules was predicted using depth residue online server or by mining of data. The binding site selection is the most important part of molecular docking, and for better results, the binding of each ligand within the active region of a target protein is required. For the interaction of ligands within the binding pocket of a target protein, the amino acids residue of PTGS2 protein were selected from His-193, Phe-196, Ser-339, Asn-368, Tyr-371, His-372, Trp-373, His-374 to Leu-376.

Molecular Docking of PTGS2

Molecular docking is an important phenomenon for observing the interaction of small molecules with proteins. In order to form stable complexes, the docking predicts the suitable orientation of ligand molecules against the target protein. The phytochemicals of *C. paradisi* leaf such as linalool, beta-pinene, and standard drug diclofenac, were used as ligand molecules against the PTGS2 protein. The beta-pinene and linalool from *C. paradisi* were docked against the PTGS2 protein using PyRx (as shown in figure IV and V). These phytochemicals were compared with the standard drug on behalf of the binding energy of each ligand. These chemical compounds were docked one by one against the PTGS2, and then collectively docked against the PTGS2. The ligand with the lowest binding energy was a more efficient therapeutic agent than other ligand molecules. The linalool from *C. paradisi* showed the lowest binding affinity (-5.5 Kcal/mol), while the binding affinity of diclofenac was -6.7 Kcal/mol. The comparison of binding energies is given in Figure **5-7.**

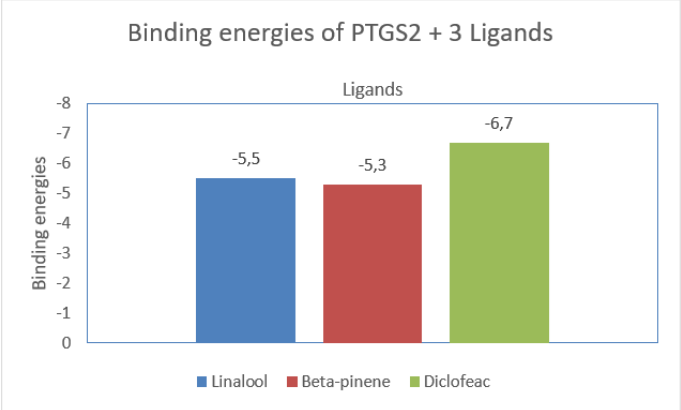

Figure 5 represents the binding energies of ligands with PTGS2 protein. Diclofenac is standard drug and linalool and beta-pinene are phytochemicals of *C. paradisi* leaf. The binding energy values of both phytochemicals are compared with the standard drug.

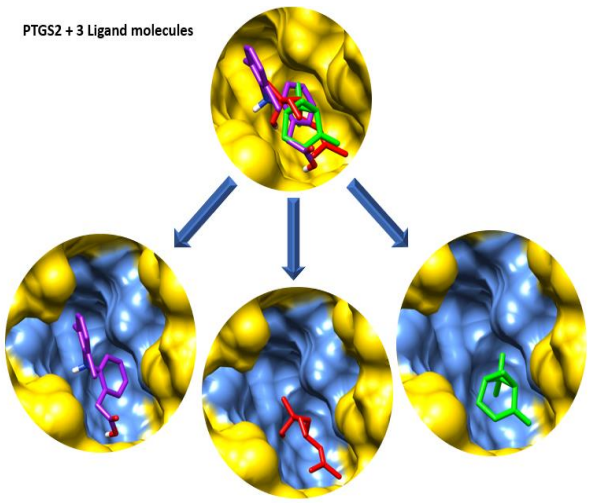

Figure 6: PTGS2 protein is represented by golden color, Binding pocket; light blue and 3 ligand molecules are represented as Linalool; red color, Beta-pinene; light green color and diclofenac; purple color.

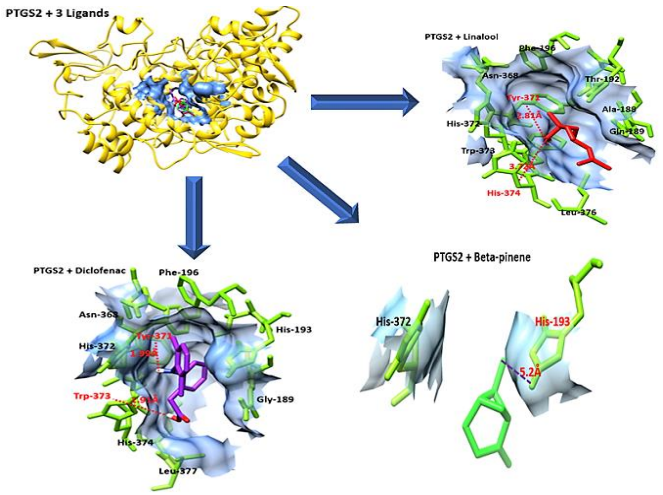

Figure 7: show the PTGS2 protein interaction with linalool, diclofenac, and beta-pinene. The linalool shows two hydrogen bonds with ptgs2 protein at Tyr-371and His-374 with bond distances 2.81Å and 3.7Å, respectively. Diclofenac with Tyr-371 and Trp-373 show two hydrogen bonds with a similar bond distance of 1.99Å. Beta-pinene shows only one hydrophobic interaction with His-193, and the bond distance was 5.2Å.

In-Silico Analgesic activity

Analgesic protein TRPV4

The protein TRPV4 (Transient receptor potential cation channel) is a member of the TRPV family linked with CNS functions, including memory, mechanical sensations, and pain. Its plays an important role in the body's normal functioning and its disturbance causes many diseases. TRPV4 is a non-selective cation receptor that has a variety of inputs such as heat, stretching, pH, endogenous ligands, and hypotonicity. TRPV4 modulating agents are used to combat various illnesses, including lung diseases, stomach conditions, cardiac insufficiency, and pain (30). The structure of TRPV4 is shown in **Figure 8**.

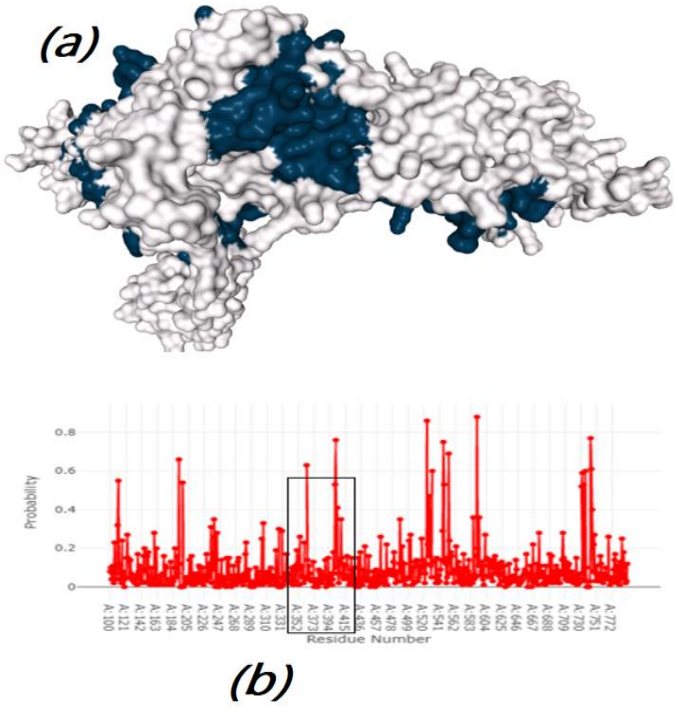

Figure 8 (a): Represent TRPV4 protein chain A with binding pocket obtained from depth residue. While in **(b)** the highlighted amino acids of binding pocket of TRPV4 protein range from 352-415 show more binding probability against phytochemicals such as beta-pinene and geraniol and diclofenac.

Binding site selection

Active site is the main region of protein for binding ligand molecules. Therefore, its selection is important for the interaction of small molecules with the target protein. The binding site of TRPV4 protein for the ligand molecules is predicted from depth residue and includes the amino acid residue from Cys-353, Ser-354, Pro-358, Asn-361, Leu-362, Glu-363, Arg-392, Glu-397, His-401, Leu-402 to Arg-404.

Molecular docking of TRPV4

Molecular docking is important to exhibit the interaction between ligand and target protein. The screening tool PyRx was used to dock the ligand-protein. It contains different parameters to give better results of docked complexes. The chemical compounds of *C. paradisi* leaf are used as ligand molecules to dock against TRPV4 protein. The beta-pinene and geraniol from *C. paradisi* and diclofenac as a standard drug were selected to dock against the target protein (TRPV4; as shown in figure 9 and 10). At first, only one ligand docked with TRPV4, and then all ligands together docked with the TRPV4.The comparison of ligand molecules shows interaction with target protein TRPV4 on the basis of binding energies of docked complexes. The lowest energy docked complex was more significant therapeutic agent as compared to other complexes. The geraniol showed the lowest binding affinity value (-5.3 Kcal/mol), that was close to the standard drug binding energy value (-5.5 Kcal/mol). The comparison of binding energies is given in Figure **9-11.**

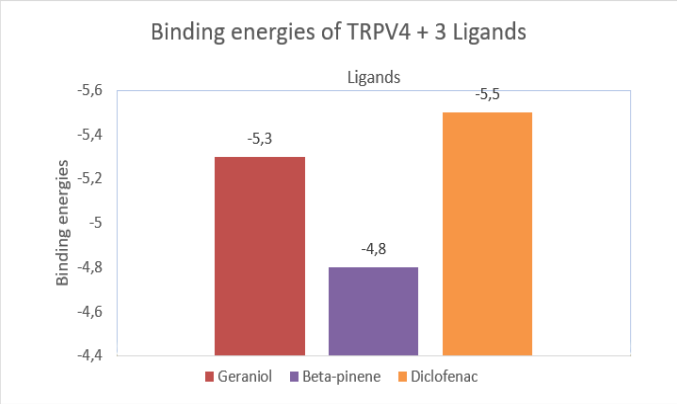

Figure 9: show the binding energies of ligand with the TRPV4 protein. The phytochemicals of *C. paradisi* leaf such as geraniol and beta-pinene binding energy values are compared with the standard drug diclofenac binding energy value. Geraniol showed more efficient result as compared to beta-pinene.

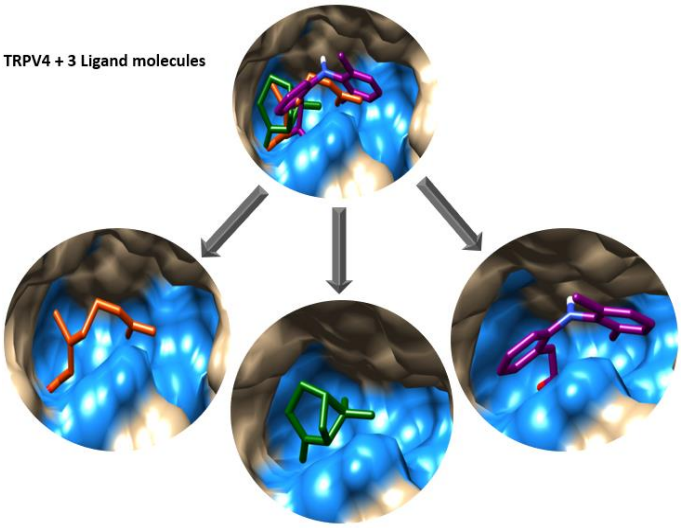

Figure 10: TRPV4 protein is represented by a tan color, Binding pocket; sky blue color and 3 ligands are represented as beta-pinene; dark green color, Diclofenac; dark magenta color and Geraniol; orange red color.

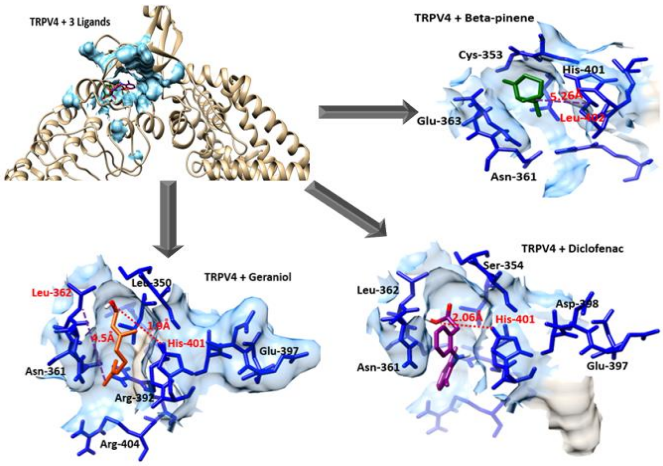

Figure 11: Show the protein TRPV4 interaction with diclofenac, beta-pinene, and geraniol. The geraniol shows one hydrogen bond with the TRPV4 protein at His-401 and one hydrophobic bond with the Leu-362 at bond distances of 1.9Å and 4.5Å, respectively. Similarly, the diclofenac shows one H-bond with TRPV4 protein at His-401 position and bond distance of 2.06Å. While beta-pinene shows hydrophobic interaction at Leu-402 with a bond distance of 5.26Å.

In-Silico Antipyretic activity

Pyretic protein TLR2

Toll-like receptors are integral glycoproteins type 1, comprised of a homologous domain (single transmembrane helix and solenoid ectodomain) of intracellular toll-interleukin-1 receptor. The ectodomain of toll-like receptor play a significant role in the identification of pathogens. TLRs may be divided into six main families and TLR2 is the part of TLR1 subfamily along with TLR1, TLR6, and TLR10. TLR2 play an important role in pathogenic infections and immune protection. When any pathogen including viruses, bacteria, fungi, and parasites enter the body, the immune system become activated against these pathogens. Normally the adaptive immune response is initiated by dendritic cells against fungal infection. These dendritic cells express TLR2, dectin-1, and TLR4 receptors that are linked with the identification of fungi. TLR2 stimulation activate the immune responses for pathogen clearance (23). The TLR2 protein structure has shown in **Figure 12**.

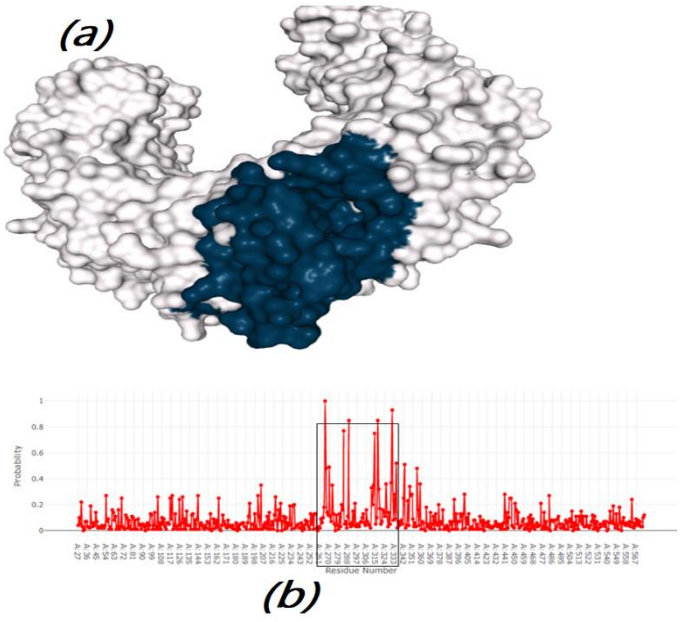

Figure 12 (a): show TLR2 protein with binding pocket obtained from depth residue. **(b)** The probability of amino acids ranges from 261-333 for binding site of TLR2 protein against ligand molecules such as citral and terpenen-4-ol.

Binding site selection

The selection of binding site is important in order to clarify the ligand interaction against target protein. The amino acid residues selected for TLR2 protein are from Leu-261, Asp-263, Phe-266, Leu-289, Val-299, Leu-317, Gln-321, Phe-322, Tyr-323, Phe-325 to Ser-333.

Molecular docking of TLR2

PyRx tool is used to dock the ligands against the target protein. *C. paradisi* phytochemical such as terpinene-4-ol and citral and standard drug paracetamol are used as ligand to dock with TLR2 protein (as shown in **Figure 14** and **15**). The result of docked complexes is compared on the basis of their binding energies. The citral show similar binding energy as paracetamol (-6 Kcal/mol). The comparison of binding energies is given in Figure **13.**

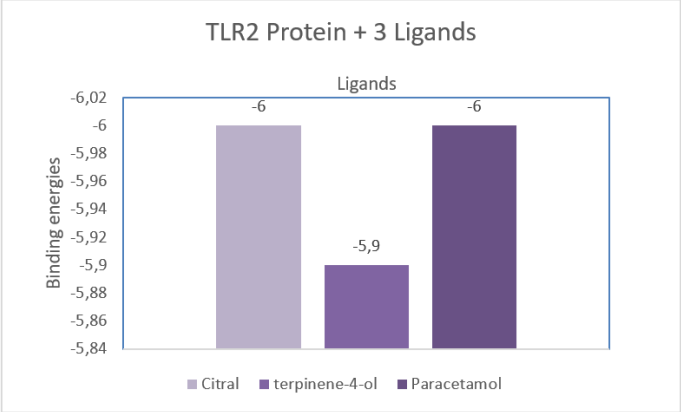

Figure 13: shows the binding energies of ligands with TLR2 protein. The citral and Terpnene-4-ol are compared with the standard drug paracetamol for binding energies values.

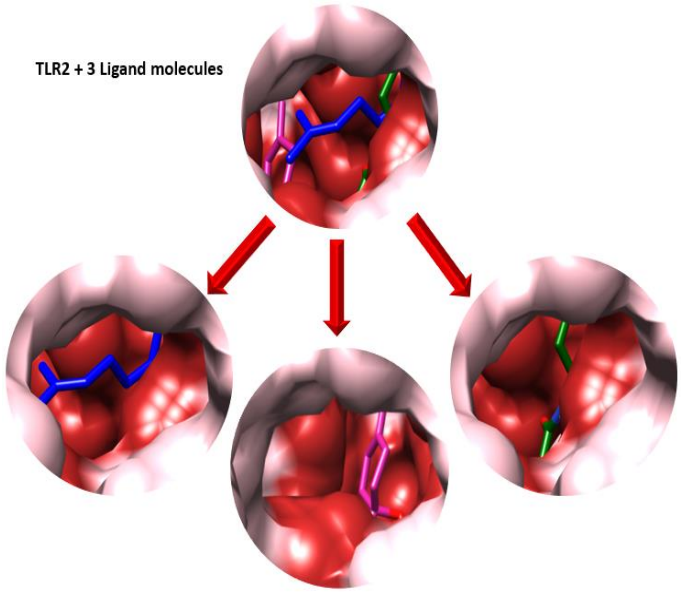

Figure 14: Protein TLR2 is represented by pink color, binding pocket; firebrick color and 3 ligands are represented as citral; blue color, paracetamol; green color and terpinene-4-ol; violet red color.

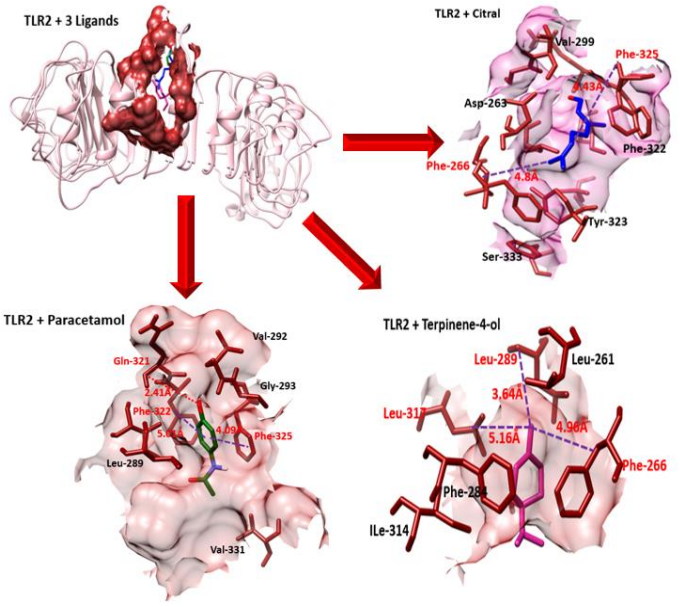

Figure 15: show the interaction of TLR2 protein with citral, terpinene-4-ol, and paracetamol. The citral show hydrophobic interaction with TLR2 protein at the position of Phe-266 and Phe-325 with bond distance of 4.8Å and 4.43Å, respectively. The terpinene-4-ol also show hydrophobic interaction with TLR2 protein at Phe-266, Leu-317, and Leu-289 and bond distance is 4.9Å, 5.16Å, and 3.64Å, respectively. The paracetamol show one H-bond at Gln-321 with bond distance 2.41Å, 2 hydrophobic interaction at Phe-322 with bond distance 5.01Å and Phe-325 with bond distance of 4.09Å.

Selected amino acids for 3 proteins

The amino acids selected for the binding interactions between ligands and 3 proteins (TRPV4, TLR2 and PTGS2) are given in the **Table 3**.

Ligands	TRPV4	TLR2	PTGS2
Linalool			Ala-188, Gln-189, Thr-192, Phe-196, Asn-368, Tyr-371, His-372, Trp-373, His-374, Leu-376
Beta-pinene	Cys-353, Asn-361, Glu-363, His-401, Leu-402		His-372, His-193, Trp-373
Diclofenac	Ser-354, Asn-361, Leu-362, Glu-397, Asp-398, His-401		Gln-189, His-193, Phe-196, Asn-368, Tyr-371, His-372, Trp-373, His-374, Leu-377
Geraniol	Leu-350, Asn-361, Leu-362, Arg-392, Glu-397, His-401, Arg-404		
Citral		Asp-263, Phe-266, Val-299, Tyr-323, Phe-322, Phe-325, Ser-333	
Terpinene-4-ol		Leu-261, Phe-266, Phe-284, Leu-289, ILe-314, Leu-317	
Paracetamol		Leu-289, Val-292, Gly-293, Gln-321, Phe-322, Phe-325, Val-331	

Discussion:

In the era of 20th century, folk medicine gained attention due to its therapeutical potential against many diseases. The plant extracts of various medicinal plants are used as analgesic, anti-pyretic, and anti-inflammatory due to the presence of many bioactive compounds (32-34). *C. paradisi* gain attention due to the presence of secondary metabolites in different parts of the plant. It contains important metabolites flavonoids, phenolic compounds, alkaloids, terpenes, saponin, and especially naringin present in the peel extract of citrus paradisi. It is used for different activities including as anti-cancer, antioxidant, anti-microbial, anti-diabetic (14-16, 35-36) . However, to our knowledge, we studied the analgesic, anti-pyretic and anti-inflammatory activity of leaf ethanolic extract of *Citrus paradisi* for the first time.

Our study evaluates that the ethanolic extract of *C. paradisi* leaf show efficient anti-pyretic and anti-inflammatory activity as compared to control group. However, the analgesic activity shows less efficient results as compared to anti-inflammatory and anti-pyretic activity. The leaf extract reduces the paw volume and pyrexia in rats induced by carrageenan and yeast. When we compared the anti-inflammatory activity with previous studies, it was proved that the bioactive compounds present in the *C. paradisi* show efficient inflammation inhibition (80%) as compared to the standard and control groups at the concentration of 200mg/kg. In 2016, a study was conducted on *C. paradisi* and *C. sinesnsis* for antioxidant and anti-inflammatory effects. Their result indicates that the juice of grapefruit and orange show efficient reduction in inflammation by increasing glutathione content (37). Glutathione removes the free radicals that cause oxidative stress and inflammation. The anti-inflammatory activity is also related to the lipoxygenase and cyclooxygenase pathways. The grapefruit juice reduce the content of myeloperoxidase, an important indicator of inflammation (37). Similarly, *Citrus aurantium* is also acknowledge due to presence of flavonoids that involve in various activities including anti-inflammatory, antioxidant, anti-cancer, antidiabetic, and anxiolytic (30). *Citrus junos* also known as Yuzu show anti-inflammatory, antidiabetic, and antioxidant effect due to presence of phytochemicals (38-42).

Then *C. paradisi* leaf extract shows less efficient analgesic activity (36% inhibition) as compared to standard group but show the result as compared to control group. This could be due to the naringenin content in mature leaf of *C. paradisi* is less than peel and seed and naringenin show analgesic activity as well as anti-inflammatory activity (43). Naringenin reduces the pain induced by acetic acid, formalin, and phenyl-p-benzoquinone. It is proved that the flavonoids present in different medicinal plants are involved in anti-inflammatory, analgesic and antioxidant activity (44). According to a recent literature review, the *Haplophyllum tuberculatum* from family Rutaceae was reported for analgesic activity (45). Similarly, *Citrus limon* and *Citrus medica* are also reported for anti-inflammatory and analgesic activity (46).

The anti-pyretic activity results were more significant compared to the control and standard groups. The leaf extract of *C. paradisi* at a high dose (400mg/kg) showed the maximum results. Previous literature support the anti-pyretic activity of *Citrus medica var limetta* (46). The anti-pyretic effect of *Clerodendrum inerm* was observed in rats and rabbits that showed the significant results in pyrexia as well as *Clitoria ternatea* root extract also showed efficient results at the dose of 50, 100, 200, and 400mg/kg in dose-dependent manner. The leaves extract of *C. paradisi* give efficient result at the high concentration 400mg/kg with 90% inhibition.

In-silico results of *C. paradisi* leaf extract for anti-inflammatory, analgesic, and anti-pyretic activity are also according to in-vivo study of these activities using proteins PTGS2, TLR2, and TRPV4. The significant results of *in-silico* anti-pyretic and anti-inflammatory activity were obtained using PTGS2 and TLR2 protein and ligand molecules such as Linalool, Beta-pinene, Citral, Terpinene-4-ol, and standard drug Diclofenac and Paracetamol. The PTGS2 protein is involved in inflammation process by inhibiting the inflammatory mediators neutrophils and macrophages (21). This protein contains cyclooxygenase that inhibits the inflammation. The beta-pinene, alpha-pinene, and linalool present in leaves of *C. paradisi* are involved in anti-inflammatory and analgesic activity (20). These molecules were selected as ligand molecules and docking of these molecules is done with anti-inflammatory PTGS2 protein. The binding affinity of beta-pinene and linalool showed moderate result as compared to standard drug diclofenac.

The computational analgesic result of analgesic activity also showed moderate result. The in-vivo result of analgesic activity show less efficient result as compared to standard group. Therefore, the binding energy of standard drug was also higher than the C. paradisi leaf components such as geraniol and beta-pinene. The protein TRPV4 is selected as analgesic protein and it is confirmed by previous literature (22, 47-53). The docking of TRPV4 was done with 2 ligand molecules (geraniol and beta-pinene) and diclofenac is used as standard drug. Similarly, citral and terpinene-4-ol were selected as ligand molecules for anti-pyretic protein TLR2. The citral is the mixture of neral and geranial that have role in anti-pyretic activity (54). The TLR2 protein is involved in anti-pyretic activity (25). The ligand molecules citral, terpinene-4-ol were selected and docked with TLR2 protein. The binding affinity of citral and terpinene-4-ol was efficient as compared to standard drug paracetamol. This indicates the therapeutical potential of these bioactive compounds against inflammation and fever (55-57).No in-silico and in-vivo evaluation of anti-inflammatory, analgesic, and anti-pyretic activity of leaf extract of C. paradisi was done before this study.

CONCLUSION

In short, the ethanolic leaf extract of *C. paradisi* can be used to reduce inflammation and fever, and to some extent, reduce pain. The results of this study indicate the more efficient antipyretic and anti-inflammatory effect of *C. paradisi* leaf extract on albino rats than the analgesic effect. Therefore, this plant can be used clinically to inhibit inflammation and fever. The reason for studying the plant *C. paradisi* is the presence of phytochemicals such as alkaloids, terpenes, phenolic compounds, saponins, tannin, and glycosides etc. To our knowledge, no previous data related to *in-silico* and *in-vivo* analysis of ethanolic leaf extract of *C. paradisi* is available. The citral, terpinene-4-ol, linalool, geraniol, and beta-pinene are the phytochemicals of leaves of *C. paradisi* show closely related binding energy compared to diclofenac and paracetamol. This indicates the therapeutical potential of these bioactive compounds against inflammation and fever. In future, further *in-silico* and *in-vivo* studies are required to understand the therapeutical potential of *C. paradisi* leaf.

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.

AUTHOR CONTRIBUTIONS

“Conceptualization, N.Z, T.F, M.H and B.Z.; methodology, A.S; software, N.Z, T.F, M.H and B.Z..; validation, A.H., and T.A.; formal analysis, A.A.S; investigation, M.A.; resources, G.N.; data curation, A.S, and G.N.; writing—original draft preparation, T.A; writing—review and editing, M.A. and A.A.S; visualization, T.A. supervision, T.A.; project administration, M.A and A.A.S.; funding acquisition, T.A.

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