

Antioxidant, Antidiabetic, Analgesic, and Antibacterial Properties of Chrysopogon zizanioides Leaf Extract: An In Vivo, In Vitro, and In Silico Evaluation

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Abstract

Chrysopogon zizanioides, commonly known as vetiver, has long been utilized in traditional medicine for its therapeutic properties. This study investigated *Chrysopogon zizanioides* leaf extract's antioxidant, antibacterial, analgesic, and antidiabetic properties. A diclofenac sodium standard and control group were used to compare the extract's analgesic impact in an animal model. The extract was 66.08% analgesic, whereas diclofenac sodium was 91.11%. Antibacterial activity was assessed against various bacterial pathogens, showing strong inhibition of gram-positive bacteria, particularly *Staphylococcus aureus*, with an inhibition zone of 30 ± 4.39 mm. The extract also demonstrated notable activity against gram-negative bacteria, with *Escherichia coli* exhibiting the highest inhibition of 22 ± 1.93 mm.

Antioxidant activity was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assays. The extract had an IC_{50} of 257.23 μ g/ml, whereas ascorbic acid had an IC_{50} of 7.8 μ g/ml. Moreover, GC-MS analysis identified 63 phytoconstituents and the antidiabetic activity showed as in silico model where in molecular docking 9,19-Cyclolanostan-3-ol acetate (3.beta.) showing the highest binding scores with proteins such as 5NN5 (-9.6820 kcal/mol) and 4GQR (-10.2851 kcal/mol). Additionally, Phytol demonstrated a Glide docking value of -9.1677 kcal/mol with protein 5F19. ADMET analysis showed the extract's non-carcinogenicity and good absorption, whereas PASS predictions and drug-likeness data suggested significant oral bioavailability, antioxidant, and anti-inflammatory properties.

These findings highlight the therapeutic potential of *Chrysopogon zizanioides* leaf extract as a natural pharmacological agent. Further research, including clinical trials and isolation of active compounds, is needed to fully understand its mechanisms and confirm its efficacy and safety for human use.

1. Introduction

Throughout an extensive duration, medicinal plants have been employed in traditional medicine to treat a wide range of ailments¹. In recent years, the focus of the study has shifted towards exploring the bioactive properties of medicinal plants². Recent research indicates that chemicals produced from plants may present viable alternatives to synthetic medications, particularly for chronic ailments such as diabetes, oxidative stress, pain control, and microbial infections. These disorders often need extended treatment periods and are associated with potential bad effects³.

Chrysopogon zizanioides, commonly known as vetiver grass, is a perennial plant belonging to the Poaceae family. It is extensively cultivated and naturalized in Asia, Africa, South America, Australia, and the Pacific region⁴. Traditionally, vetiver is primarily grown in riverside areas, and different parts of the plant have varied pharmaceutical applications. The root extracts are valued for their anti-inflammatory, antioxidant, and antibacterial properties. The leaf juice is used to treat parasitic infestations when administered orally, and also serves as animal feed and compost⁵. However, research on the leaves of *C. zizanioides* is limited compared to other parts of the plant. Emerging studies are beginning to uncover the potential benefits of the leaves, which have often been overlooked.

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia or elevated blood glucose levels. Various plant extracts have demonstrated hypoglycemic effects by modulating glucose absorption, stimulating insulin release, and inhibiting α -amylase activity. Although specific research on the antidiabetic effects of *C. zizanioides* is scarce, it is important to recognize its potential in diabetes treatment. Preliminary investigations undertaken by experts have revealed that certain plant extracts, particularly those rich in phenolic chemicals, may have the potential to demonstrate antidiabetic benefits⁶.

Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, is the root cause of several clinical disorders⁷. Plant extracts contain various antioxidants like phenolic compounds, flavonoids, and carotenoids, which neutralize free radicals and prevent oxidative damage to biomolecules. Studies have shown that *C. zizanioides* exhibits significant antioxidant activity, scavenging free radicals, inhibiting lipid peroxidation, and protecting cells from oxidative damage. This antioxidant capacity is attributed to the presence of phytochemicals such as vetiverol, khusimol, and elemicin⁸. Moreover, the quest for innovative antimicrobial drugs has become imperative in response to the growing prevalence of antibiotic resistance. Previous research has documented the antibacterial properties of *C. zizanioides* against a range of pathogenic bacteria, and the fundamental mechanisms entail the rupture of bacterial cell membranes and the interference with metabolic activities⁹. Furthermore, analgesic activity has been observed in several plant extracts, revealing various mechanisms. These mechanisms encompass the suppression of inflammatory mediators and the adjustment of pain pathways. Still, researchers have not completely investigated the analgesic properties of *C. zizanioides*. Considering the existence of substances with anti-inflammatory capabilities, it is reasonable to speculate that the plant extract could possess pain-relieving benefits¹⁰. Additionally, molecular docking simulations have shown possible binding affinities between the bioactive components of this substance and important protein targets associated with oxidative stress, diabetes, pain, and bacterial infections. In addition, quantitative structure-activity relationship (QSAR) models have been used to forecast the bioactivity of structurally similar compounds, making it easier to find new lead molecules. Thereby aiding in the creation of safe and effective phytopharmaceuticals from *C. zizanioides*.

This study investigates *C. zizanioides* leaf extract's antioxidant, antidiabetic, analgesic, and antibacterial activities using in vivo, in vitro, and in silico methods. In vitro testing will focus on oxidative stress mitigation, while in vivo animal models will assess the extract's efficacy in diabetes and pain treatment. In silico molecular docking studies will elucidate the molecular interactions and mechanisms of action. The research aims to explore the diverse biological activities of *C. zizanioides* leaf extract, contributing to the evidence supporting the medicinal potential of natural products. The findings may pave the way for new therapeutics utilizing bioactive compounds from medicinal plants.

2. Results

2.1 In vitro antioxidant effect

The extract from *C. zizanioides* leaves exhibited a DPPH free radical scavenging activity of 94.39% at a concentration of 200 $\mu\text{g/mL}$, whereas the leaf extract and ascorbic acid demonstrated activities of 89.10% and 89.10%, respectively (**Figure 1**). The IC_{50} values for the leaves extract and ascorbic acid were 257.23 $\mu\text{g/mL}$ and 7.8 $\mu\text{g/mL}$, respectively. The results suggest that the leaf extract exhibited a modest level of antioxidant activity in comparison to the control group, which was treated with ascorbic acid.

2.2 Analgesic test

When given orally at a dosage of 500 mg/kg body weight, the extract resulted in a 58.8% decrease in writhing in mice. In comparison, the conventional medicine diclofenac sodium exhibited a 91.11% inhibition at a dosage of 10 mg/kg body weight. (**Table 1**).

2.3 Antibacterial test

C. zizanioides extract exhibited antibacterial activity against both gram-positive and gram-negative bacteria. The zones of inhibition for *C. zizanioides* were smaller than those of Ciprofloxacin, a broad-spectrum antibiotic used as a control, for all tested bacteria. However, the extract created an inhibition zone 30 ± 4.39 mm, 14 ± 4.39 mm, 8 ± 4.39 mm against *Staphylococcus aureus*, *Vibrio mimicus*, *Bacillus megaterium* against gram positive bacteria. It also demonstrated activity against other gram-negative bacteria with a zone of inhibition 22 ± 1.93 mm, 16 ± 1.93 mm, 15 ± 1.93 mm against *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi* bacteria (**Figure 2**). These findings suggest that *C. zizanioides* extract possesses potential antibacterial properties, although further research is needed to determine the minimum inhibitory concentration (MIC) and identify the specific compounds responsible for this activity.

2.4 Molecular docking study

In our investigation, we employed Glide docking to achieve precise docking, scoring, and prediction of ligand binding modalities to receptors (**Table 2**). The compound 9,19-Cyclolanostan-3-ol acetate (3β) demonstrated strong binding with the receptors 4GQR, 5NN5, and 4GQQ compared to other ligands, indicating superior antidiabetic activity with 5NN5 and 4GQR (**Figure 3**), and enhanced antibacterial activity with 4GQQ (**Figure 4**). The human pancreatic α -amylase (RCSB ID: 4GQR), crucial for starch hydrolysis, is a major therapeutic target for type II diabetes. Additionally, 9,19-Cyclolanostan-3-ol acetate (3β) showed notable binding affinity with glutathione peroxidase (RCSB ID: 1GP1), human erythrocyte catalase (RCSB ID: 1QQW), and human superoxide dismutase (RCSB ID: 2C9V), indicating significant potential for antioxidant activity (**Figure 5**). Phytol, an acyclic diterpene alcohol and a chlorophyll constituent, also exhibited strong binding to Human Cyclooxygenase-2 (RCSB ID: 5F19), a protein involved in inflammation, suggesting its potential for analgesic effects (**Figure 6**). Docking of 9,19-Cyclolanostan-3-ol acetate (3β) against the proteins 5NN5, 4GQR, 4GQQ, 1QQW, 2C9V, and 1GP1 resulted in docking scores of -9.6820, -10.2851, -11.994, -11.4604, -9.8821, and -11.2322 kcal/mol, respectively

and Phytol, while docking with the protein 5F19, achieved a docking score of -9.1677 kcal/mol, suggesting its potential for analgesic effects. Molecular docking studies showed that lower (more negative) docking scores reflect stronger binding affinities, indicating a higher potential for biological activity. Additionally, stronger binding usually correlated with greater in vivo potency, as the compound was more likely to effectively inhibit or modulate the target protein.

9,19-Cyclolanostan-3-ol acetate (3 β) formed specific interactions with various proteins. With 5NN5, it established one alkyl bond with ILE174 (5.40 Å) and two hydrogen bonds with ASN148 (2.97 Å) and ASP95 (3.44 Å). In complex with 4GQR, 3 β created three alkyl bonds with MET278 (5.45 Å), TYR319 (4.73 Å), and TYR323 (4.66 Å), one pi-alkyl bond with TYR276 (2.84 Å), and one hydrogen bond with TYR323 (3.60 Å). The 4GQQ complex included two hydrogen bonds with ARG195 (2.76 Å) and ARG337 (3.03 Å), one alkyl bond with LEU162 (4.13 Å), and five pi-alkyl bonds with HIS15 (4.84 Å), PHE17 (5.05 Å), TRP58 (5.17 Å), TYR62 (5.01 Å), and HIS201 (4.84 Å). Interaction with 2C9V involved two alkyl bonds with CYS111 (3.58 Å) and ILE113 (4.93 Å). The 1QQW complex displayed six alkyl bonds with ARG127 (5.12 Å, 4.90 Å), LYS177 (4.88 Å), ALA251 (3.38 Å), VAL182 (4.66 Å), and VAL247 (4.98 Å), two hydrogen bonds with GLY118 (3.31 Å) and LYS177 (3.26 Å), and one pi-alkyl bond with HIS466 (4.36 Å). Lastly, in the 1GP1 complex, 3 β formed six alkyl bonds with ALA21 (4.45 Å), LEU89 (4.48 Å), PRO103 (5.38 Å), LYS93 (5.42 Å), LEU20 (4.77 Å), and LEU107 (5.12 Å), and one hydrogen bond with ASN90 (3.04 Å). Phytol, when bound to 5F19, formed six pi-alkyl bonds with PHE205 (4.96 Å), PHE209 (5.21 Å), PHE381 (4.99 Å), TYR385 (4.40 Å), TRP387 (5.12 Å), and PHE518 (5.15 Å), along with four alkyl bonds with VAL349 (4.43 Å), LEU384 (5.15 Å), LEU352 (4.07 Å), and LEU534 (3.77 Å).

2.5 ADMET study

A comprehensive assessment of the pharmacokinetic properties of the substances—including toxicity, excretion, metabolism, distribution, and absorption—was conducted using the admetSAR web server (**Table 3**). This server employs cut-off scores to evaluate these attributes, and all selected ligands have a relative molar mass of less than 500 Daltons. Human intestinal absorption data, which are crucial for initial ligand evaluation, indicates all compounds show excellent human intestinal absorption, with values near or at 1.0000. This suggests that these compounds are likely to be well-absorbed when administered orally¹¹. Additionally, the ability of these compounds to cross the blood-brain barrier is significant for maintaining brain homeostasis¹². The ligands shown favorable outcomes with regards to their capacity to pass across the blood-brain barrier, inhibition of P-glycoprotein which is favorable for avoiding drug-drug interactions mediated by P-glycoprotein, and be absorbed by the human intestines.

Further analysis of cancer-causing potential and toxicity revealed that none of the compounds are expected to have adverse effects, though Neophytadiene(0.4862) was noted for potential carcinogenicity. AMES toxicity test, with values close to 0.9, indicating a low likelihood of mutagenicity and, therefore, low genotoxic risk. The evaluation of hERG inhibition, critical for generating cardiac action potentials, showed that lack of strong hERG inhibition across all compounds suggests a low risk of inducing cardiac arrhythmias, suggesting that all are suitable for further laboratory testing.

Drug-likeness was assessed using the Lipinski Rule of Five criteria via the pkCSM server (**Table 4**). Although some drugs, particularly those for cancer treatment, may not meet all criteria, compounds should preferably possess a molecular weight that is lower than 500 Da, ≤ 5 hydrogen bond donors, ≤ 10 hydrogen bond acceptors, and a logarithm of the partition coefficient (logP) below 5. Though, all the compounds meet the molecular weight, hydrogen bond donor, and acceptor criteria of the Lipinski Rule of Five, their high AlogP values suggest that they may face challenges with solubility and, consequently, oral bioavailability. The AlogP values, indicative of lipophilicity, vary significantly across the compounds, ranging from 5.7489 (9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)) to 8.8196 (.gamma.-Sitosterol). Notably, all compounds have AlogP values exceeding the preferred maximum of 5, suggesting that while these high values indicate favorable properties for crossing biological membranes, the compounds' poor solubility, and potential for low bioavailability present significant challenges due to their high lipophilicity, which might influence their absorption and distribution properties.

The ADMET and Drug-likeness analysis showed that these compounds had excellent intestinal absorption, favorable blood-brain barrier permeability, and low toxicity. However, their high AlogP values suggested poor solubility and reduced oral bioavailability, which might limit their effectiveness as oral drugs. Finally, by balancing lipophilicity with improved solubility and bioavailability is crucial for their development as viable therapeutic agents.

2.6 PASS Prediction

The PASS algorithm accurately predicted biological activities with 90% accuracy based on analyzing structure-activity relationships (**Table 5**). PASS (Prediction of Activity Spectra for Substances) predictions use the terms Pa and Pi to describe the likelihood that a compound will display a certain biological activity. The Pa, or Probability of Activity, indicates how likely the compound will show the predicted activity, with values ranging from 0 to 1. A higher Pa value suggests a stronger chance that the compound will have the desired effect. Conversely, Pi, or Probability of Inactivity, represents the probability that the compound will not exhibit the specified activity. Pi also ranges from 0 to 1, and a higher value implies a greater likelihood that the compound lacks the predicted activity. The relationship between Pa and Pi helps assess confidence in the prediction, with a higher Pa typically signaling a greater likelihood of the compound being active. According to the PASS prediction, Phytol emerges as a standout compound with high predicted activity in antioxidant (Pa of 0.475 and Pi of 0.008) and antibacterial (Pa of 0.417 and Pi of 0.026) areas, while 9,12,15-Octadecatrienoic acid, methyl ester and Neophytadiene show strong potential in antidiabetic (Pa of 0.400 and Pi of 0.013) and antioxidant areas, respectively. 13-Docosenamide has the highest probability of being an analgesic stimulant with a Pa of 0.326 and a Pi of 0.020, while other compounds exhibit a range of activities, with varying degrees of potential across different biological effects.

2.6 GC-MS screening

The GC/MS analysis of the extract has identified 63 compounds based on their retention durations, and their structures have been validated using the NIST Library of chemicals (**Table S1**) (**Figure 7**). The

chemicals that have been discovered are shown together with their retention duration, area percentage, molecular weight, and molecular formula (**Table 6**). According to Table S1, there were thirteen esters, three hydrocarbons, two sesquiterpenes, and five alcohol. The chemical found in the extract is 9,19-Cyclolanostan-3-ol, acetate, (3.beta.), having a peak area of 8.8%. followed by 13-Docosenamide, (Z)- (8.35%), 1,4-Benzenedicarboxylic acid, bis(2- ethylhexyl) ester (7.01%), .gamma.-Sitosterol (5.20%), Neodiosgenin (3.beta.,25S) acetate (4.8%), 15.alpha.-Hydroxyculmorin (4.03%) etc. are the majorly occurring compounds.

3. Discussion

Phytochemicals produced from plants have received considerable interest as possible medication candidates and pharmacological therapies for fighting infectious illnesses¹³. Numerous plant parts exhibit diverse pharmacological properties, including antioxidant, anticancer, anti-tuberculosis, antidepressant, and antimicrobial activities^{14–16}. *C. zizanoides* is believed to owe its biological effects to a wide array of bioactive phytoconstituents, such as fatty acids, flavonoids, carbohydrates, anthocyanin, cardiac glycosides, phenols, and alkaloids¹⁷. Our comprehensive analysis revealed that the ethanolic leaf extract of *C. zizanoides* possesses moderate to high antioxidant, antibacterial, and antidiabetic properties. Additionally, the extract is rich in antimicrobial phytoconstituents, including alkaloids, glycosides, flavonoids, phenolic compounds, and esters. Moreover, GC-MS analysis identified 63 phytochemicals in the leaf extract, with 9,19-Cyclolanostan-3-ol, acetate, (3.beta.)- and 13-Docosenamide, (Z)- as the most abundant compounds.

The ethanolic leaf extract demonstrated potent antibacterial activity against various bacterial strains, particularly Gram-negative bacteria like *Escherichia coli*, *Salmonella typhi*, and *Salmonella paratyphi*. Furthermore, antioxidants effectively counteract DPPH radicals by donating hydrogen. Given the pivotal role of free radicals in biological damage, the DPPH assay is widely used to assess the antioxidant capacity of medicinal plants¹⁸. The methanol extract demonstrated potential antioxidant activity, consistent with the known free radical scavenging properties of phenolic compounds like tannins^{19,20}. While the root of *C. zizanoides* has shown effective antioxidant activity in previous studies^{21–24}, our focus on the leaf extract revealed moderate antioxidant properties.

Molecular docking was employed to investigate ligand-protein interactions at the atomic level, providing insights into drug molecule behavior within specific protein binding regions where binding affinity, a quantitative measure of complex stability, was assessed using docking scores^{25,26}. More negative values indicate stronger binding affinity between ligand and protein. In this study, 9,19-Cyclolanostan-3-ol, acetate, (3.beta.) exhibited the highest binding affinity for antidiabetic, antibacterial, and antioxidant activities, while Phytol demonstrated the highest affinity for analgesic activity. Additionally, docking analysis revealed nonbonded interactions between 9,19-Cyclolanostan-3-ol, acetate, (3.beta.), and proteins 5NN5, 4GQR, 4GQQ, 1QQW, 2C9V, and 1GP1, as well as between Phytol and the 5F19 protein within their respective active sites. In addition, ADMET prediction identified potential drug candidates

based on desired pharmacokinetic properties. However, Neophytadiene raised concerns regarding carcinogenicity.

Some compounds deviated from Lipinski's Rule of Five, but most had favorable pharmacological characteristics. Lipinski's Rule suggests keeping the AlogP value below 5 to ensure good solubility and permeability. Since all the compounds exceed this value, they are highly lipophilic, which could lead to poor water solubility and difficulties in drug formulation and absorption. The PASS prediction evaluation highlights Phytol as a standout compound with exceptional antioxidant and antibacterial properties. 13-Docosenamide emerges as a promising candidate for analgesic stimulant activity, while 9,12,15-Octadecatrienoic acid, methyl ester demonstrates significant potential for antidiabetic effects.

Despite promising results, this study has limitations such as, the antibacterial activity was assessed against a limited number of bacterial strains, necessitating a broader evaluation. The antioxidant activity of the ethanolic leaf extract was moderate compared to standard antioxidants, warranting further research to enhance its potential. While GC-MS analysis identified 63 phytochemicals, their contributions to biological activities remain unexplored. This study relied on in vitro assays, and in vivo studies are essential to validate findings and assess pharmacokinetics, bioavailability, and toxicity. Additionally, the influence of geographic location, soil conditions, and harvesting time on phytochemical composition requires further investigation. Optimal concentrations and dosages for therapeutic applications also need to be determined.

4. Materials and Methods

4.1 Plant material

The leaves of *C. zizanoides* were obtained at Sadullahpur, Kotalipara, Gopalganj, Bangladesh. A voucher specimen was then authenticated by the scientific officials at the Bangladesh National Herbarium in Mirpur, Dhaka, and assigned the accession number DACB-104521.

4.2 Preparation for extraction

The gathered plants were rinsed with clean water, chopped into little fragments, and air-dried for a duration of two weeks. The desiccated botanical matter was pulverised into a fine powdery state. The plant extract was obtained by a cold extraction process, where 200 mg of powdered plant material was mixed with 700 mL of ethanol in a glass container for a duration of 14 days. The extract was isolated from the plant waste by filtration using Whatman filter paper. The extract was condensed by the process of evaporation, initially through open air and finally through a water bath. The extract yielded 8.77%.

For the preparation of the extraction process, the plant leaves were dried using a cold extraction process and followed by ground into fine powdered form. Then the 875ml ethanol solvents from Merck (Merck®, Kenilworth, NJ, USA) were dissolved with 250g fine powders of plant extract for around 14 days in a

glass container. Evaporating under a controlled temperature around 50°C the crude turned into dark-green ethanol residue 16g.

4.3 Test animals

Swiss albino mice of either sex (20-29g) were collected from Jahangir Nagar University animal housing lab, and housed at the Pharmacology Lab at Bangladesh University, Dhaka, Bangladesh. Mice were housed in individually ventilated cages under specific pathogen-free, maintained in alternating 12-hour light/12-hour dark cycles, and fed with food and water.

4.4 GC-MS analysis

The leaves of *C. zizanioides* were used to prepare an ethanolic extract. This extract was then analyzed using gas chromatography-mass spectrometry (GC-MS) with an Agilent instrument from the United States. The analysis was performed using a High Performance-5MS 5% Phenyl Methyl Silox capillary column, which had dimensions of 30m length, 250 µm I.d., and 0.25 µm film thickness. The electron impact system exhibited an ionization energy of 70 electron volts (eV) at a source temperature of 230°C. Helium gas with a high level of purity was employed at a consistent flow rate of 1.00 ml/min. The oven temperature was originally set at 50°C for 1 minute, then increased to 200°C for 2 minutes, and eventually raised to 300°C for 7 minutes. The mass spectra of the volatile components from the study were identified by matching them with the MS data library of the National Institute of Standards and Technology (NIST 08.L) connected to the GC-MS instrument, leading to the identification of bioactive compounds.

4.5 In vitro antioxidant test using the DPPH method

The antioxidant properties of the powerful cytotoxic extract from *C. zizanioides* leaves were assessed using a stable DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay. The assay followed the established method with minor adjustments^{27,28}, with ascorbic acid used as the reference compound. Stock solutions of ascorbic acid and each fraction (1000 µg/ml) were prepared in ethanol and serially diluted to obtain concentrations ranging from 0.977 to 500 µg/ml. A DPPH solution (5 mg/250 ml ethanol) was prepared, and 3.0 ml aliquots of each were combined with 2.0 ml aliquots of each concentration of standard or fraction solution. The extent of the reaction in each mixture was evaluated by measuring its absorbance at 517 nm following a 30-minute incubation at room temperature in the absence of light, with ethanol serving as a control.

The percentage inhibition of free radical production was determined using the formula:

$$\% \text{ DPPH inhibition} = [(A - B)/A] \times 100$$

Here, A represents as blank solution, and B signifies as extract solution.

4.6 Analgesic assay

Writhe is a term that refers to the process in which an animal's abdomen constricts, causing the entire body to elongate and the hind limbs to extend²⁹. The analgesic effect of the sample was evaluated using the acetic acid-induced writhing test in mice³⁰. The animals were randomly divided into three groups: control, positive control, and test, with three mice in each group. The control group received 1% Tween-80 in water at a dose of 10 mL per kilogram of body weight. The positive control group was given diclofenac sodium at a dose of 25 mg per kilogram of body weight. The test group received the sample at a dose of 500 mg per kilogram of body weight. All treatments, including the control vehicle, standard drug, and sample extracts, were administered orally 30 minutes before the intraperitoneal injection of 0.7% acetic acid. After a 5-minute absorption period, the number of writhing episodes was recorded over a 15-minute duration. The analgesic activity was quantified by calculating the percentage inhibition of writhes in the rats treated with the extract compared to the control group. The number of writhing in the control was taken as 100% and percent inhibition was calculated as follows:

% Inhibition of writhing = $100 - (\text{treated mean} / \text{control mean}) \times 100$

4.7 Antibacterial assay

The antibacterial activity was assessed using the methodology outlined by Nostro et al. (2000)³¹. Both gram-positive and gram-negative bacteria starting *Staphylococcus aureus*, *Bacillus megaterium*, *Bacillus subtilis*, *Vibrio mimicus*, *Bacillus cereus*, *Salmonella paratyphi*, *Shigella boydii*, *Salmonella typhi*, *Escherichia coli*. The Whatman no. 1 filter paper was sliced into circular discs with a diameter of 6 mm and subjected to sterilization. Next, begin with extracts that have been adjusted to a final concentration of 400 µg, dissolved in 10% v/v DMSO. These discs, along with blank discs as negative controls, and Ciprofloxacin (5mcg/disc) was used as a control. These were placed on nutrient agar plates pre-inoculated with the respective test microorganisms. Following a 24-hour incubation at 4°C to enable diffusion of the test substance, bacterial growth was assessed via a subsequent 24-hour incubation at 37°C. The antibacterial activity of the extract was determined by measuring the diameter of inhibition zones surrounding the sample discs³².

4.8 In silico assay

4.8.1 Molecular docking preparation

The 3D conformation of the protein was made available in PDB format through the RCSB Protein Data Bank³³. The structures of antidiabetic proteins α-glucosidase and α-amylase (PDB IDs: 4GQR, 5NN5), an antibacterial protein thymidylate kinase (PDB ID: 4GQQ), antioxidant proteins SOD (PDB ID: 1SOD), catalase (PDB ID: 1QQW), and glutathione peroxidase (PDB ID: 1GP1), and an analgesic protein COX2 (PDB ID: 5IKQ) were included.

GLIDE (Grid-Based Ligand Docking with Energetics), a molecular docking program developed by Schrödinger, was selected for its robust accuracy in predicting protein-ligand interactions and its ability to efficiently screen large compound libraries. LigPrep, part of Schrödinger's suite, was used to process

ligands including 9,12,15-Octadecatrienoic acid, methyl ester,(Z,Z,Z), 9,19-Cyclolanostan-3-ol acetate, (3.β.), 13-Docosenamide, neophytadiene, phytol, gamma-sitosterol, and 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester. The procedure included adding hydrogen atoms, removing ionic compounds, and incorporating ions at pH 7 ± 2.0 . The protein preparation module was used to construct 3D structures of both native and mutant proteins, which involved completing missing side chains, capping termini, generating disulfide bonds, adding hydrogen molecules, and establishing bond orders³⁴. Energy minimization was performed using the OPLS3e force field³⁵.

A grid box was constructed around the proteins' known binding sites. Default parameters for van der Waals scaling (1.0) and charge cutoff (0.25) were used to determine the grid size. The Ligand Docking program with GLIDE XP parameters was used for docking. A scaling factor of 0.80 adjusted the van der Waals radius, and a score threshold of 0.15 was applied as a standard criterion³⁶. The docking score formula used was:

$$\text{Docking score} = \text{avdW} + \text{bCoul} + \text{Hbond} + \text{Metal} + \text{Lipo} + \text{BuryP} + \text{RotB} + \text{Site}$$

In this context, 'a' and 'b' represent the coefficients for van der Waals (vdW) and Coulomb (Coul) energies, respectively. 'Hbond' and 'Metal' refer to the protein's hydrogen bonding and metal association capabilities. 'Lipo' indicates lipophilicity, 'BuryP' denotes the penalty for buried polar groups, 'RotB' stands for the penalty for rotatable bonds, and 'Site' describes polar interactions at the active site³⁷.

4.8.2 ADMET prediction and PASS prediction

The ADMET characteristics of small compounds were assessed using the admetSAR (<http://lmmd.ecust.edu.cn/admetsar2/>) website. ADMET property prediction is facilitated by the advanced multitask graph neural network framework (CLMGraph). Modern machine learning techniques are implemented by the website to create algorithms for prediction that encompass the key ADMET characteristics for the search for drugs. The ligands' canonical SMILES have been collected from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) website³⁸. The adherence of molecules to Lipinski's rule of 5 was calculated using the PKCSM website and the PASS online tool was used to estimate the biological activity profiles of drug-like organic compounds. This guideline evaluates whether a chemical substance with specific pharmacological activity has the physical and chemical properties needed to be an orally active medication in humans³⁹. However, while ADMET and PASS prediction models use historical data and machine learning algorithms, they may not fully account for the complexities of drug interactions and metabolism in vivo. Therefore, predictions should be supplemented with empirical testing to validate the computational results.

4.9 Statistical analyses

All data were analyzed using Graph Pad Prism (version 10.3.0) statistical software. Results are presented as mean $\pm$ SEM (standard error of the mean), with p values less than 0.05 considered

significant. Statistical significance was assessed with a one-way ANOVA (analysis of variance), followed by Dunnett's test for comparisons to the control group.

5. Conclusions

The study of *C. zizanioides* leaf extract has shown considerable promise as an antidiabetic, antibacterial, analgesic, and antioxidant agent. Analgesic tests on animal models indicated promising activity, with specific data revealing a notable reduction in pain comparable to standard analgesics. While the antioxidant and antibacterial effects were moderately effective, these findings suggest preliminary evidence of the extract's therapeutic potential. The study identified that 9,19-Cyclolanostan-3-ol acetate (3 beta) exhibited the highest binding affinities with targeted antidiabetic, antibacterial, and antioxidant proteins, while Phytol compounds showed the greatest affinities with targeted analgesic proteins, supported by specific binding affinity values.

Given the moderately effective antioxidant and antibacterial results, the extract's potential should be viewed with caution, emphasizing the preliminary nature of these findings. The results highlight the importance of further research and clinical trials to validate these effects in humans, with a focus on dosage determination, potential side effects, and interactions with existing treatments. The study underscores the relevance of *C. zizanioides* in modern phytotherapy and ethnopharmacology, suggesting that this plant could serve as a model for discovering other plant-based therapeutics. Additionally, it opens avenues for future research in ethnopharmacology, particularly in exploring the traditional uses of other under-researched medicinal plants.

Overall, the study supports the traditional use of *C. zizanioides* in herbal medicine and emphasizes the need for further investigation to isolate and characterize the active compounds responsible for these effects. Integrating this natural extract into modern medical practices could offer an effective, natural alternative, contributing to the advancement of phytotherapy and ethnopharmacology.

Abbreviations

Chrysopogon zizanioides, *C. zizanioides*; Liquid chromatography–mass spectrometry, GC-MS; Adsorption distribution metabolism excretion toxicity, ADMET; 1,1-Diphenyl-2-picrylhydrazyl, DPPH; Prediction of Activity Spectra for Substances, PASS; Sajidur Rahman Akash, SRA; Most. Afrin Akter, MAA; Chayan Talukder, CT; Sumaya Alam Mim, SAM; Md. Abdullah Al Obaid, MAAO; Md. Atikur Rahman, MAR; Mirajul Islam, MI; Jahidul Islam Himu, JIH; Tareq Aziz, TA; M A Rashid, MAR; Mst. Lubna Jahan, MLJ; Md. Sarafat Ali, MSA

Declarations

6. Conflict of interest

There is no conflict of interest in this research.

7. Acknowledgements

The authors dedicate this paper to all the students who sacrificed their lives for the quota movement that happened in 2024. We are proud of their bravery in fighting for their rights. Additionally, the authors acknowledge the Department of Pharmacy at Bangladesh University for their lab support in this research, especially Ohidul Anik for his guidance during the experiments, and Gazi Sabri Reza Abir for his contributions.

8. Author contribution

Methodology, Writing: SRA, MAA, SAM; Experiment analysis: SRA, CT, MI; Validation: SRA, MAA; Review: SRA, MAAO, MAR, JIH, TA, MLJ, MSA, MAR; Supervision: MLJ, MSA. The final version of this paper was approved by all authors.

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10. Supplementary file

There is an extra file added as a supplement containing additional data on this manuscript.

11. Ethical approval

Ethical approval for this study was granted by the Department of Pharmacy, State University of Bangladesh Research Ethics Committee under reference number 2024-04-29/SUB/A-ERC/001.

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Tables

Tables 1 to 6 are available in the Supplementary Files section

Figures

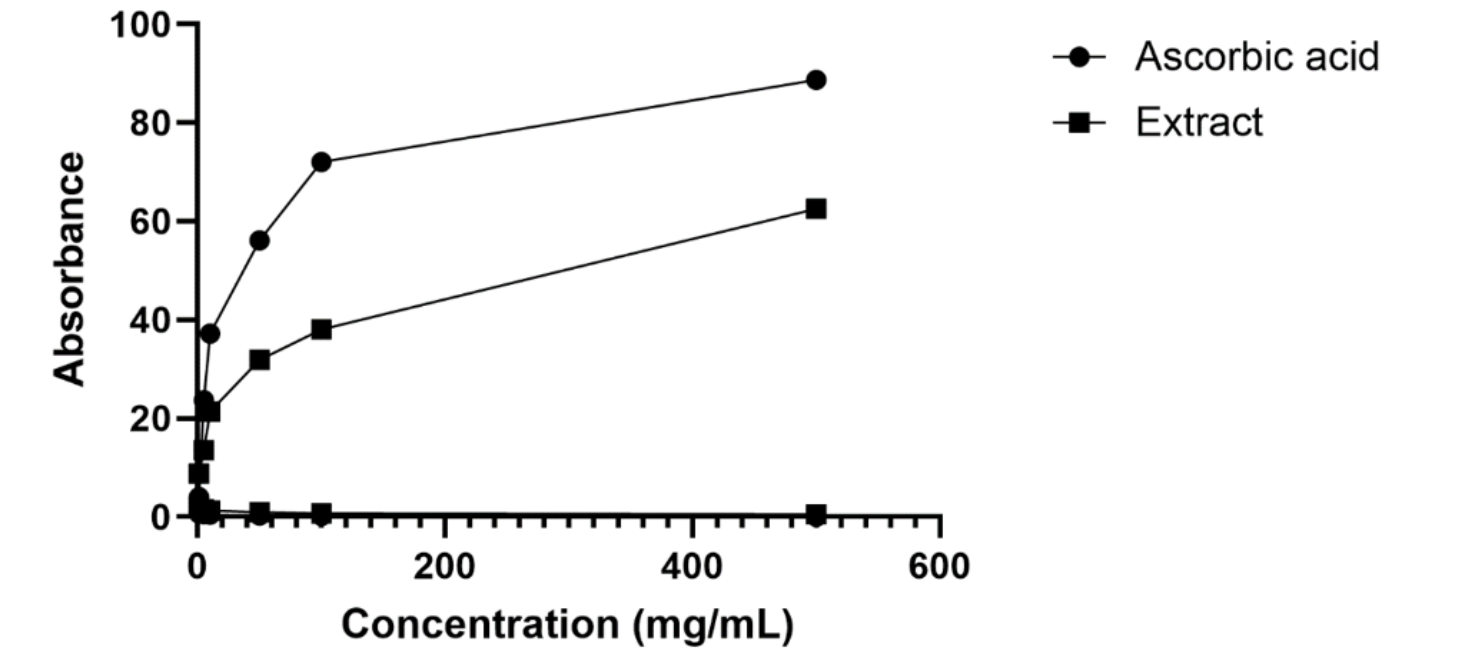


Figure 1

Antioxidant activity of *C. zizanioides* leaf extract compared to ascorbic acid (positive control) as determined by DPPH radical scavenging assay. Results are expressed as percentage inhibition of DPPH radical.

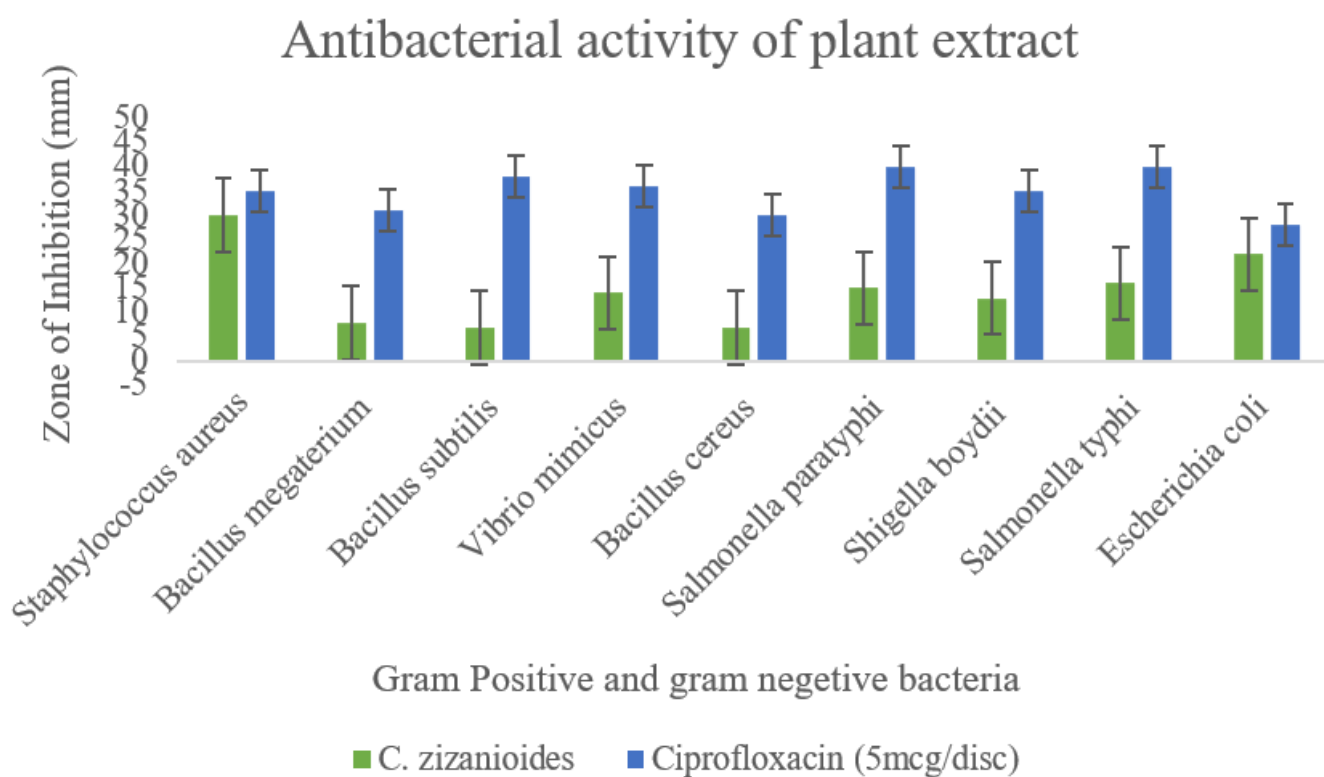


Figure 2

The antibacterial activity of *C. zizanioides* leaf extract was tested against several gram-positive and gram-negative bacteria where the value reported as the mean $\pm$ SEM of many replications, showed that a $P < 0.05$ indicated a considerable degree of activity.

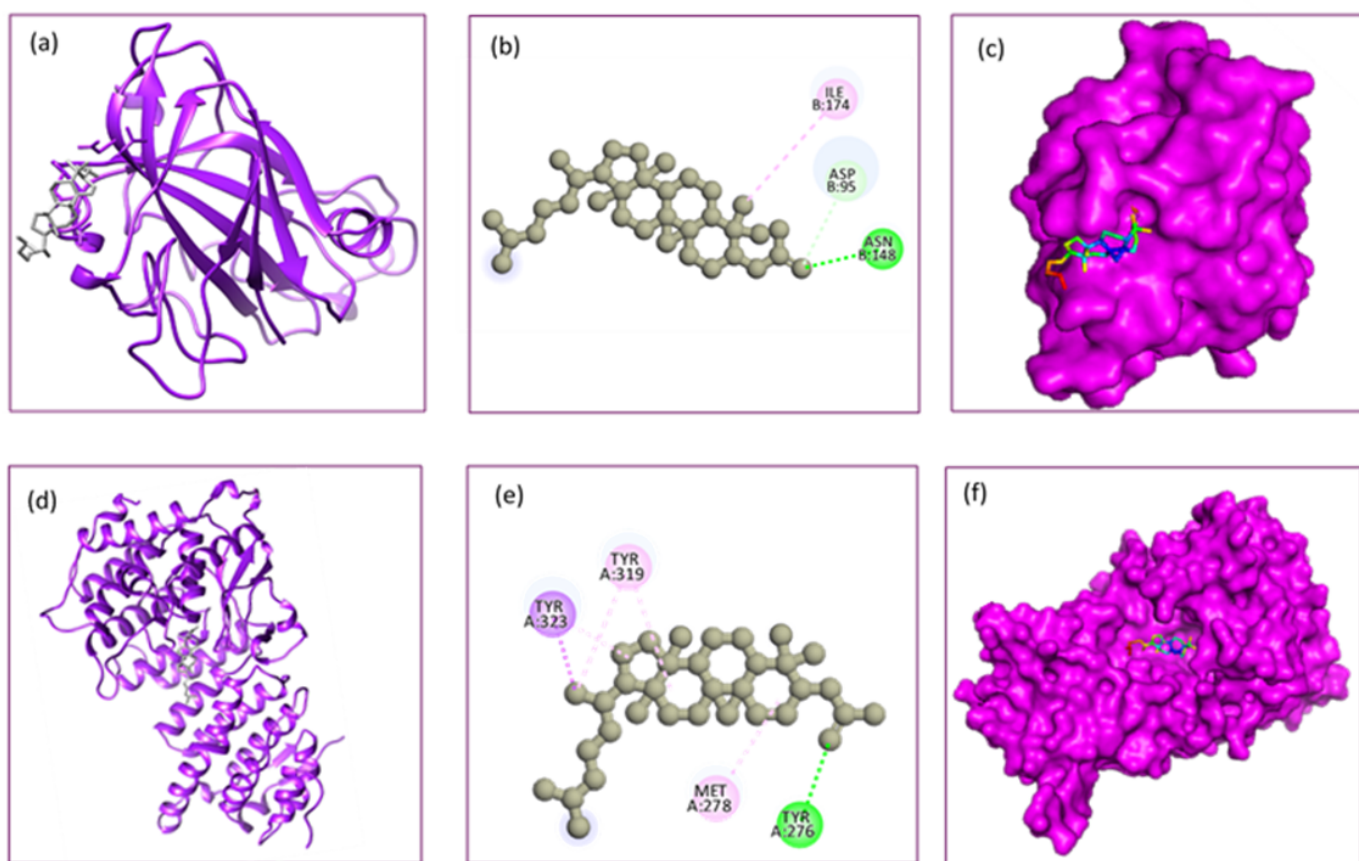


Figure 3

Schematic representation of 5NN5-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) and 4GQR-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex. (a) Pose view of 5NN5-9,19-Cyclolanostan-3-ol,acetate, (3.beta.) complex (b) 2D interaction of 5NN5-9,19-Cyclolanostan-3-ol,acetate (3.beta.) complex (c) Surface view of 5NN5-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex. (d) Pose view of 4GQR-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex (e) 2D interaction of 4GQR-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex (f) Surface view of 4GQR-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex.

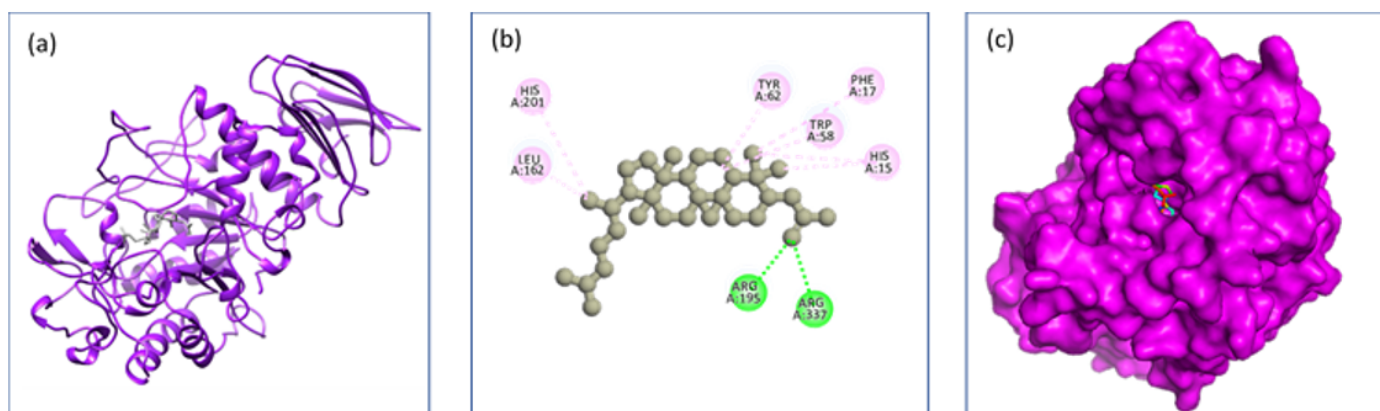


Figure 4

Schematic representation of 4GQQ-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex. (a) Pose view of 4GQQ-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex (b) 2D interaction of 4GQQ-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex (c) Surface view of 4GQQ-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex.

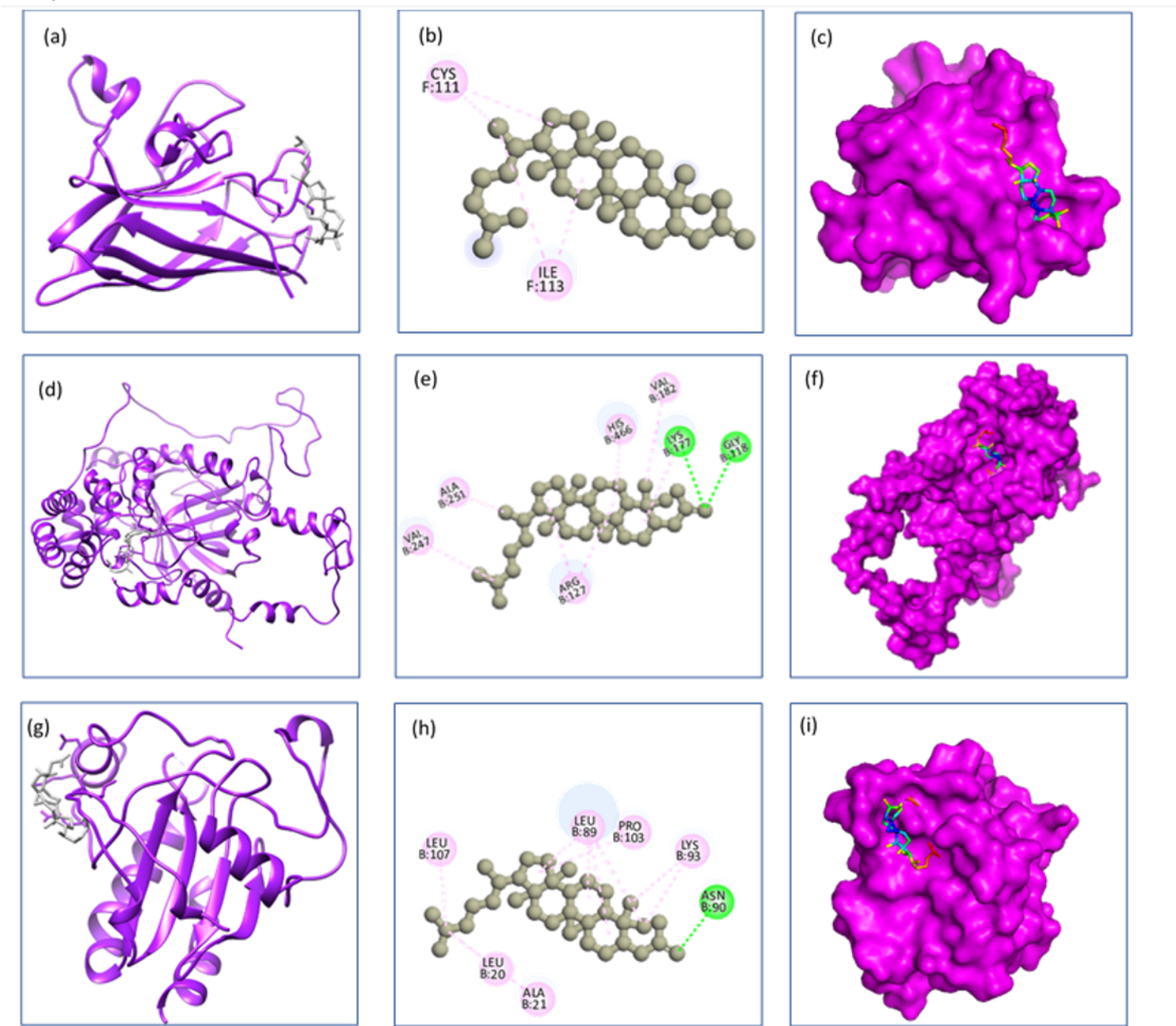


Figure 5

Schematic representation of 2C9V-9,19-Cyclolanostan-3-ol,acetate,(3.beta.), 1QQW-9,19-Cyclolanostan-3-ol, acetate,(3.beta.), and 1GP1-9,19-Cyclolanostan-3-ol,acetate ,(3.beta.) complex. (a) Pose view of 2C9V-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex (b) 2D interaction of 2C9V-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex (c) Surface view of 2C9V-9,19-Cyclolanostan-3-ol,acetate,(3.beta.) complex. (d) Pose view of 1QQW-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex (e) 2D interaction of 1QQW-9,19-Cyclolanostan-3-ol, acetate,(3.beta.) complex (f) Surface view of 1QQW-9,19-Cyclolanostan-3-ol,

acetate,(3.beta.) complex (g) Pose view of 1GP1-9,19-Cyclolanostan-3-ol,acetate ,(3.beta.) complex (h) 2D interaction of 1GP1-9,19-Cyclolanostan-3-ol,acetate ,(3.beta.) complex (i) Surface view of 1GP1-9,19-Cyclolanostan-3-ol,acetate ,(3.beta.) complex.

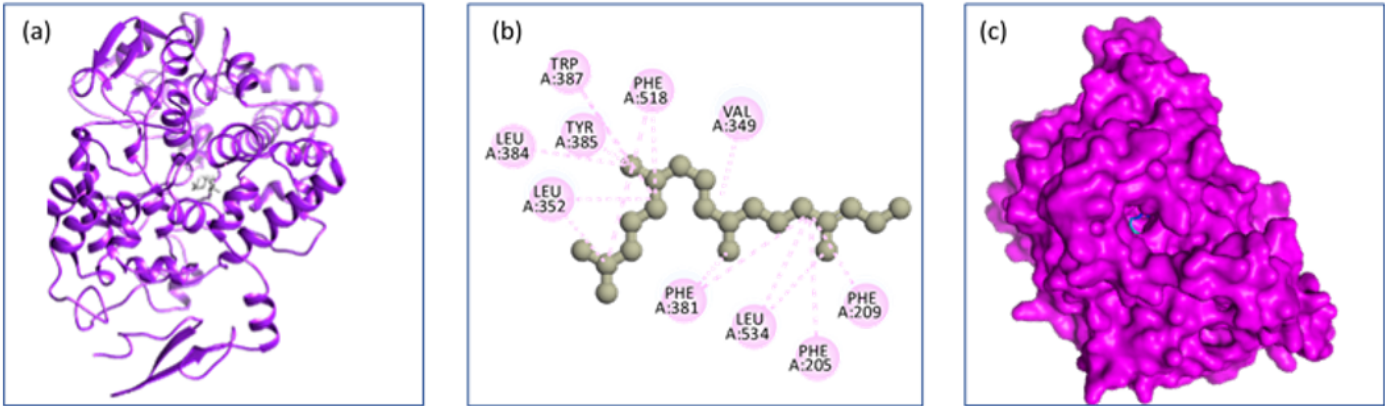


Figure 6

Schematic representation of 5F19-Phytol complex. (a) Pose view of 5F19-Phytol complex (b) 2D interaction of 5F19-Phytol complex (c) Surface view of 5F19-Phytol complex.

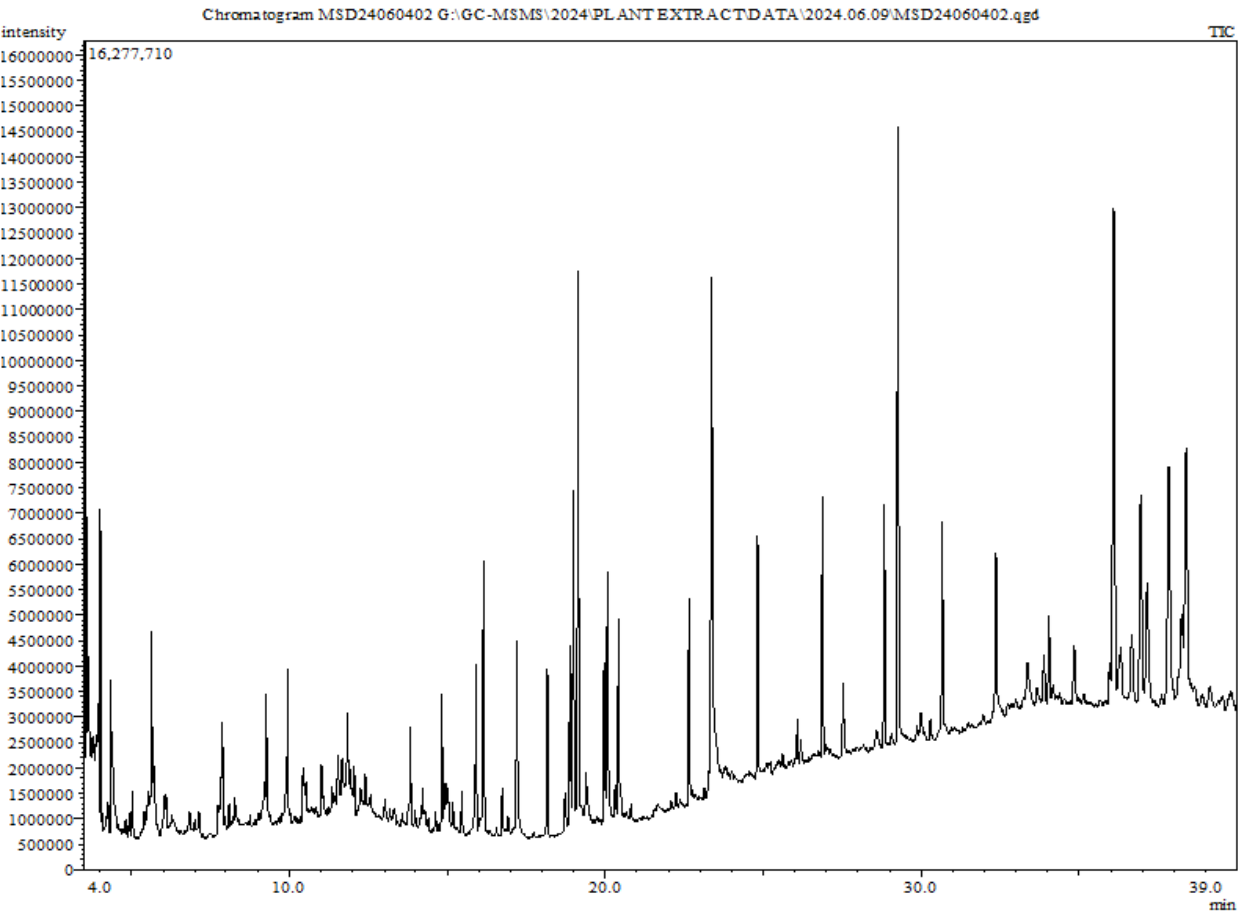


Figure 7

Ethanollic extract of *C. zizanioides* leaves by GC-MS chromatography.

Supplementary Files

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