A Comparative Analysis of Bioactive Phytochemicals in Cynanchum viminale (L.) L. and Pergularia daemia (Forssk.) Chiov. from Saudi Arabian Folk Medicine

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Research Article

Keywords: C. viminale, P. daemia, Medicinal plants, Phytoconstituents, Bioactivity

Posted Date: October 8th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7674030/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at The Science of Nature on December 11th, 2025. See the published version at https://doi.org/10.1007/s00114-025-02039-2.

Abstract

The dual global health crises of antimicrobial resistance and cancer demand the urgent discovery of novel therapeutic leads. The medicinal plants *Cynanchum viminale* and *Pergularia daemia* (Apocynaceae) represent a rich reservoir of bioactive compounds used in traditional medicine, yet a comprehensive comparative analysis of their bioactivities and molecular mechanisms has remained elusive. This study provides an integrated in vitro and *in silico* analysis of the methanolic extracts of *C. viminale* and *P. daemia*. Phytochemical profiles were delineated by HPLC. Bioactivities were systematically evaluated via a battery of five antioxidant assays (DPPH, ABTS, FRAP, H_2O_2, NO), broad-spectrum antimicrobial screening against six pathogens, and cytotoxicity assays against a panel of six human cancer cell lines (HepG-2, MCF-7, HCT-116, A-549, PC-3, A-431). To deconstruct the molecular basis of these activities, in silico molecular docking was performed against two pivotal therapeutic targets: Epidermal Growth Factor Receptor (EGFR) and Dihydrofolate Reductase (DHFR). A striking functional divergence between the two plants was discovered. *P. daemia* exhibited superior antioxidant and broader-spectrum antibacterial activity, a finding strongly correlated with its unique phenolic profile, particularly the potent DHFR-binding Chlorogenic acid and the bioenhancing alkaloid Piperine. In stark contrast, *C. viminale* demonstrated dramatically superior cytotoxic potency across all tested cancer cell lines, with IC_{50} values as low as 24.37 µg/mL against HepG-2 liver cancer. Molecular docking brilliantly illuminated the mechanism behind this divergence: the potent cytotoxicity of C. viminale is driven by its principal alkaloids, Protopine and Berberine, which showed high-affinity binding to the ATP-binding site of EGFR, a key driver of cancer proliferation. DHFR, conversely, was identified as a common molecular target for potent binders from both plants (Evodiamine, Piperine, Chlorogenic acid), providing a unifying mechanism for their shared antimicrobial properties.

1. Introduction

Wild plants are good sources of novel natural compounds that could have distinct therapeutic targets. Among these plants, Apocynaceae family is now becoming known for being a reliable source of several unique compounds that can save lives [1]. This family is one of the most important angiosperm families and has been used worldwide for many therapeutic purposes. It is rich in alkaloids, triterpenoids, cardenolides, and iridoids, which have a variety of biological and pharmacological implications, including cardioprotection, hepatoprotection, neuroprotection, anticancer, anti-inflammatory, and antimalarial activities. Additional ingredients, including lignans, sterols, lactones, and sugars have been identified in this family and thoroughly investigated. Apocynaceae species are used to treat gastrointestinal problems, pain, malaria, fever, diabetes, skin ailments and ectoparasitic diseases in conventional medicine[2]. Several plants in this family are utilized as food by many tribal communities, while a few of them are poisonous[3]

Cynanchum viminale L. (synonym: Sarcostemma viminale) and *Pergularia daemia* (Forssk.) Chiov. belong to the family Apocynaceae and have long been used in Asian folk medicine[4] [5]. *Cynanchum viminale*, also known as a caustic vine, is used frequently in traditional medicine to cure cancer and has

toxic effects on livestock [6]. From this plant, several phenolic compounds, flavonoids and lignans have previously been reported. Moreover, seco-pregnane aglycones cynavimigenin A and cynaviminoside A, as well as novel pregnane glycosides cynaviminoside B and cynavimigenin B compounds, were isolated from this species. These compounds demonstrated limited cytotoxicity against cancer and normal human cell lines [6]. The *C. viminale* aqueous leaf extract possesses antipyretic, anti-inflammatory, and analgesic properties in albino mice, while the ethyl acetate extract of *C. viminale* demonstrates notable anti-inflammatory properties and potent anti-psychotic activity, as well as an inhibitory effect on the central nervous system. The plant's decoction is used as a gargle for gonorrhea, muscle pain, and infections of the mouth and throat.

P. daemia is rich in phytoconstituents, which are used to cure a variety of ailments. It also plays an important role in natural drug research since it has many in vitro and in vivo pharmaceutical actions, such as calming inflammation. Moreover, it possesses antiarthritic, antimicrobial, antifertility, and cytotoxic properties [5]. Triterpenes, flavonoids, saponins, and sterols are among the metabolic components of *P. daemia* [7]. According to [8], *P. daemia* leaf extract has antibacterial activity against *Proteus vulgaris*, *Staphylococcus aureus*, and *Bacillus subtilis*.

C. viminale and P. daemia are native to Saudi Arabia[9] (IPNI, 2024) and they are widely spread in different dry and wet habitats throughout the country. C. viminale latex is used to cure wounds in Saudi Arabian traditional medicine [10], whereas *P. daemia* is used to treat jaundice, liver illnesses, and inflammatory problems by Saudis[1]. Based on this background, the study aimed to assess the phytoconstituents and biological activity of methanol extracts from *C. viminale and P. daemia*, with goal of providing scientific support for their use in traditional medicine.

2. Materials and methods

2.1. Plant materials and extraction

Plant samples of *C. viminale and P. daemia* were collected from various habitats across Saudi Arabia. The entire shoot system of each plant was dried at room temperature for one month, and then ground into a coarse powder using an electric grinder. The powdered samples were defatted with petroleum ether (40–60°C) for 12 hours, then filtered and dried. One hundred grams of each plant sample was macerated in 1000 mL of methanol and shaken with an orbital shaker at 150 rpm for 72 hours. After filtration, the filtrate was dried using a rotary evaporator (Buchi, USA).

2.2. Determination of total polyphenols

The spectrophotometric assay, was applied to determine the total polyphenols in each plant. Five mL of water was mixed with 0.5 mL Folin-Ciocalteu reagent, 0.1 ml (0.5 mg/mL) sample then left at room temperature for 15 min. Subsequently, 25% Na_2CO_3 (1.5 mL) was added to the mixture, which was then allowed to stand for 30 min at 40°C. Absorbance was measured at 765 nm using a Jenway UV-6420 Uv-

Vis spectrophotometer. Gallic acid was chosen as the reference standard, and concentrations were expressed as milligram gallic acid equivalent per g dry weight (mg GaE/g DW).

Determination of total flavonoids

In a test tube, plant samples (0.5 mg/mL, 2.4 mL) were mixed with $NaNO_2$ (0.05g/mL, 0.3 mL), then NaOH (0.04 g/mL, 4 mL) was added. The mixture was allowed to stand at room temperature for 15 min. The absorbance was read at 510 nm, and quercetin was used as the standard flavonoid. Total flavonoid content was expressed as mg quercetin equivalent per g dry weight (mg QE/ g DW).

2.3. Determination of total tannins

The method of[11] was used to quantify the total tannins in each sample. Plant extract (0.5 mg/mL, 1 mL) was added to a vanillin/methanol solution (4%, 3 mL) followed by the addition of concentrated HCL (1.5 mL). After 30 min at 25°C, the absorbance was read at 500 nm. Tannin concentration was expressed as mg equivalent of tannic acid per g dry weight (mg TAE/ g DW).

2.4. Determination of total alkaloids

HCL (2N, 10 mL) was used to dissolve the plant samples (0.5 mg). After filtration, one mL of the filtrate was taken into a separatory funnel, washed with chloroform, and the pH was adjusted to 7.0 using NaOH (0.1 N). Bromocresol green (10^{-4} M, 5 ml) and phosphate buffer (pH 4.7, 5 mL) were added to the slurry and then mixed. The formed complex was extracted with 1,2,3, and 4 mL of chloroform, then collected in a volumetric flask (10 mL) and diluted with chloroform. The absorbance was measured at 417 nm. The concentration of alkaloid was expressed as mg atropine per g dry weight (mg AT/ g DW).

2.5. HPLC analysis

To determine individual phenolic acid and flavonoid compounds, each plant sample (50 mg/mL) was analyzed by HPLC (Agilent 1100) equipped with two LC-pump, a UV-Vis detector and a C18 column (125 mm ×4.60 mm, 5 µm particle size). Chromatographic separation of the target compounds was carried out using Agilent ChemStation. With phenolic acids were separated using a detection wavelength of 250 nm and a gradient mobile phase of two solvents: Solvent A (methanol) and Solvent B (acetic acid in water 1:25). Flavonoid compounds were separated using an isocratic elution (70:30) procedure with acetonitrile (A) and 0.2% (v/v) aqueous formic acid (B) as the mobile phase. The gradient program started with 100% solvent B, maintaining this concentration for the first 3 min. This was followed by a 5-min period with 50% eluent A. The concentration of eluent A was then increased to 80% for the next 2 min, before being reduced back to 50% for the remaining 5 min of the experiment. The constructed standard curve was used to detect the target compounds, and the amounts were expressed as mg/g dry weight.

2.6. Antioxidant assays

2.6.1. DPPH radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazl) method[12] was applied to evaluate the DPPH radical scavenging capacity of each extract. Three mL of DPPH solution (100 mmol L^{-1}) were mixed well with plant sample (0.5 mg/mL, 0.05 mL) and methanol (4 mL). After incubation in the dark for 60 min at 25°C, the absorbance was measured at 515 nm. The radical scavenging capacity of each plant sample was calculated using the equation below. The antioxidant capacity was presented as an IC_{50} value (µg/mL).

$$\% \text{ DPPH radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c = Control absorbance and A_s = Sample absorbance.

2.6.2. ABTS radical scavenging assay

ABTS (2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) radical assay[13] was performed to assess the ABTS radical scavenging activity in each sample. A 0.04 mL aliquot (0.5 mg/mL) of each plant extract was dissolved in 1.8 mL of ABTS reagent (7 mM), and the mixture was diluted to a total volume of 4 mL with methanol. After incubation in the dark at °C, the absorbance at 734 nm was recorded. The antioxidant activity was determined using the equation below and given as an IC_{50} value (µg/mL).

$$\% \text{ ABTS radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

A_c = Control absorbance and A_s = Sample absorbance.

2.6.3. Hydrogen peroxide assay (H2O2)

The outlined method[14] was adopted to measure the capacity of the two extracts to scavenge the hydrogen peroxide radical in the prepared solution. Ten mL of hydrogen peroxide reagent (40 mM) were made in a phosphate buffer (50 mM, pH 7.4) and stored in a brown glass container. A one-mL aliquot of each extract was thoroughly mixed with the hydrogen peroxide reagent and the reaction medium was allowed to remain at room temperature for 15 min. The absorbance was detected at 230 nm. The ability of each extract to scavenge hydrogen peroxide was determined using the below equation and the value was expressed as an IC_{50} (µg/mL).

$$\% \text{ } H_2O_2 \text{ radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

2.6.4. Nitric oxide radical scavenging assay (NO)

The modified procedure stated by[15] was adopted to evaluate the nitric oxide scavenging activity of the tested samples. Each sample (0.5 mg/mL, 0.5 mL) was mixed with a prepared solution from sodium nitroprusside (10 mM) in 50 mM phosphate buffer saline (pH 7.4). After 15 min at room temperature, 0.5 mL of Griess reagent which was prepared by mixing one mL of sulfonic acid (33% in 20% glacial acetic acid) with one mL of 0.1% naphthalene diamine dihydrochloride (w/v) was added to the mixture. The absorbance was recorded at 540 nm. The formula below was used to calculate the percentage of nitric oxide scavenging capacity of the plant extracts and given as an IC_{50} value (µg/mL).

$$\%\ \text{nitric oxide radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

2.7. Determination of cytotoxicity

2.7.1. Cell culture

The following cell lines were obtained from ATCC in United States: HepG-2 (human hepatocellular carcinoma), MCF-7 (human breast adenocarcinoma), HCT-116 (human colon cancer), A-549 (lung adenocarcinoma), PC-3 (human prostate cancer), A-431 (epidermoid carcinoma), and MRC-5 (Normal human foetal lung fibroblast). Cancer cells were maintained in RPMI-1640 medium [10% fetal bovine serum (FBS), L glutamine, and 1% antibiotic-antimycotic]. The normal cell (MRC-5) was grown in Eagle's minimum essential medium (EMEM, 10% FBS). The tested cells were grown at 37°C, 5% CO_2, 95% air and 100% relative humidity.

2.7.2. Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to assess the cytotoxicity of the two plant samples [16]. A hundred microliter of each suspension was taken into 69-well (3×10^3 cells/well) plates and allowed to stand for 12 h at 37°C. Each extract had the following concentrations in media (DMSO, 0.1%): 0, 0.05, 0.5, 5, 25, 50 µg/mL. After three days, MTT reagent (10 µL, 5 mg/mL) was added to each well. The optical density was read at 570 nm and the IC_{50} values were calculated using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA. Cell lines were maintained in appropriate media (DMEM/RPMI-1640) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2. Cytotoxicity was assessed using the MTT assay as described by Mosmann (1983). Cells were seeded in 96-well plates (1×10^4 cells/well) and treated with serial dilutions of extracts (0.25–500 µg/mL) for 48 h. Doxorubicin served as the positive control. Absorbance was measured at 570 nm, and cell viability was calculated as:

Viability (%)=(*A*sample ⁄ *A*control)×100

IC_{50} values (concentration causing 50% inhibition of cell growth) and CC_{50} values (concentration causing 50% cytotoxicity in normal cells) were determined via non-linear regression (GraphPad Prism v9.0). All

experiments were performed in triplicate with three independent replicates. The selectivity index (SI) was calculated as: SI=(CC50 on normal cells/ IC50 on cancer cells). Higher SI values indicate greater selectivity for cancer cells over normal cells.

2.8. Antimicrobial assay

2.8.1. Test organisms

antimicrobial activity was tested using four bacterial strains: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NRRL B-543, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, and two fungal species: *Aspergillus fumigatus* ATCC 13073 and *Candida albicans* ATCC 10231.

2.8.2. Well diffusion method

Antimicrobial activity was evaluated by the procedure proposed by [17]. Plates of potato dextrose agar were seeded with a 16-hour suspensions of fungal spores, and plates of nutrient agar were seeded with an 8-hour broth culture of different bacteria. Using a sterile cork borer, two wells (10 mm) were made on each plate. Plant extract (100 µL, 10 mg/mL) was placed into the wells and left at room temperature for 2 h. The plates were kept at 37 for 24 h and 72 h for bacteria and fungi, respectively. The test was repeated three times and inhibition zone (mm) was measured. Gentamycin (4µg/ml) and Ketoconazole (100 µg/ml) were used as positive controls for bacteria and fungi, respectively.

2.9. Molecular Docking Analysis

All molecular docking simulations and preparations were conducted using the AutoDock 4.2.6 suite, which includes AutoDock Tools (ADT) 1.5.6 for file preparation[18]. The core docking calculations were performed using AutoDock Vina 1.1.2, renowned for its accuracy and computational efficiency. Post-docking analysis and visualization of molecular interactions were carried out using Biovia Discovery Studio Visualizer v21.1.0. The crystal structures of the target proteins, human Epidermal Growth Factor Receptor (EGFR) kinase domain in complex with Erlotinib (PDB ID: 4HJO) and Dihydrofolate Reductase (DHFR) in complex with NADPH (PDB ID: 3FY8), were retrieved from the RCSB Protein Data Bank. The three-dimensional (3D) structures of the principal alkaloid and phenolic compounds identified in the *C. viminale* and *P. daemia* extracts were obtained from the PubChem compound database. The chemical structures were optimized to achieve their lowest energy conformation using the MMFF94 force field[19]. Using ADT, the prepared ligands were then converted to the PDBQT format. This automated process defined the rotatable bonds within each ligand, allowing for conformational flexibility during the docking simulation, and assigned the necessary Gasteiger charges. AutoDock Vina generated nine distinct binding poses for each ligand, ranked by their predicted binding energy (BE) in kcal/mol. To validate the accuracy and reliability of the docking protocol, a re-docking procedure was performed. The original co-crystallized ligands (Erlotinib for 4HJO and NADPH for 3FY8) were extracted and then docked back into their respective receptor active sites using the identical protocol described above. The predicted binding pose was then superimposed onto the original crystallographic pose, and the Root Mean Square Deviation (RMSD) was calculated. An RMSD value of less than 2.0 Å is the gold standard

for a successful validation, indicating that the docking protocol can accurately reproduce the experimentally determined binding mode. Our validation yielded RMSD values well within this threshold, confirming the reliability of our computational setup.

1.1. Statistical analysis

Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc multiple comparison test, utilizing Graph Pad prism software version 5.0 Results were considered statistically significant at a p-value of ≤ 0.05. Data are presented as mean ± SD, with n = 3.

3. Results and discussion

3.1. Comprehensive HPLC Analysis of Bioactive Alkaloids and Phenolics in *Cynanchum viminale* and *Pergularia daemia.*

This work provides a detailed analysis of HPLC chromatographic data for the methanol extracts of two medicinally significant Apocynaceae species: *Cynanchum viminale* (L.) L. and *Pergularia daemia* (Forssk.) Chiov. The objective is to characterize and compare their profiles of key alkaloidal and phenolic constituents, compounds renowned for their diverse pharmacological activities, including antioxidant, anticancer, and antimicrobial effects[20]. This analysis serves to provide a chemical basis for the traditional uses of these plants and to identify potential leads for further pharmacological investigation.

3.1.1. Phytochemical Profile of *Cynanchum viminale.*

The HPLC analysis of the *C. viminale* extract delineates a unique combination of phenolic compounds and alkaloids (Table 1).

Phenolic Compounds

The chromatogram for phenolics displays five well-resolved peaks against a stable baseline, indicating an effective separation method. The identified compounds include **Catechol** (RT 4.0 min, 12.69 µg/g), **Cinnamic acid** (RT 6.8 min, 7.45 µg/g), **Pyrogallol** (RT 8.6 min, 5.13 µg/g), **Gallic acid** (RT 10.0 min, 8.05 µg/g), and **Salicylic acid** (RT 11.8 min, 7.63 µg/g). **Catechol** is the most abundant among these identified phenols. The presence of these compounds, particularly **salicylic acid**, **gallic acid**, and **catechol**, contributes to the plant's known anti-inflammatory and antioxidant properties[21] [22].

Table 1
Comparative alkaloid and phenolic composition of *C. viminale* and *P. daemia* methanol extracts

Plant Species	RT (min)	Compound	Conc.	Significance
Alkaloid Profiling				
C. viminale	3.0	Evodiamine	5.26 µg/mg	Cytotoxic agent
	4.0	Berberine	1.46 µg/mg	Antimicrobial, anticancer
	8.0	Protopine	8.95 µg/mg	Antispasmodic, cholagogue
	10.0	**Cycloclavine**	**6.09 µg/mg**	**Major compound**
P. daemia	5.0	Piperine	11.23 µg/gm	Bioavailability enhancer
	7.0	Tetrandrine	2.59 µg/gm	Anticancer, anti-inflammatory
	9.0	**Camptothecin**	**10.44 µg/gm**	Potent topoisomerase I inhibitor
	9.8	Cycloclavine	1.09 µg/gm	
Phenol Profiling				
C. viminale	4.0	**Catechol**	**12.69 µg/gm**	Major compound; Antioxidant
	6.8	Cinnamic acid	7.45 µg/gm	Antimicrobial
	8.6	Pyrogallol	5.13 µg/gm	Antioxidant
	10.0	Gallic acid	8.05 µg/gm	Antioxidant, anticancer
	11.8	Salicylic acid	7.63 µg/gm	Anti-inflammatory
P. daemia	3.0	Chlorogenic acid	2.52 µg/gm	Antioxidant, hepatoprotective
	5.0	Syringic acid	2.89 µg/gm	Antioxidant, anticancer
	7.0	Cinnamic acid	6.24 µg/gm	Antimicrobial
	8.9	**Pyrogallol**	**10.79 µg/gm**	Major compound; Antioxidant
	9.8	Gallic acid	5.42 µg/gm	Antioxidant, anticancer

Alkaloid Content: The alkaloid analysis of *C. viminale* reveals a different set of compounds: **Evodiamine** (RT 3.0 min, 5.26 µg/mg), **Berberine** (RT 4.0 min, 1.46 µg/mg), Protopine (RT 8.0 min, 8.95 µg/mg), and **Cycloclavine** (RT 10.0 min, 6.09 µg/mg). Protopine, a widely studied benzylisoquinoline alkaloid, is the most prominent in this extract and is recognized for a broad range of biological activities, including anti-inflammatory, analgesic, and anticancer effects[23] [24]. The presence of berberine further adds to the extract's potential therapeutic value, as it is known for its potent antimicrobial and metabolic-regulating effects.

3.1.2. Phytochemical Profile of *Pergularia daemia.*

The HPLC analysis for *P. daemia* demonstrates a phytochemical signature that is markedly different from that of *C. viminale* (**Table-1**), which directly correlates with its distinct bioactivities[1] [25].

- **Phenolic Compounds**: The chromatogram identifies five key phenolics: **Chlorogenic acid** (RT 3.0 min, 2.52 µg/g), **Syringic acid** (RT 5.0 min, 2.89 µg/g), **Cinnamic acid** (RT 7.0 min, 6.24 µg/g), **Pyrogallol** (RT 8.9 min, 10.79 µg/g), and Gallic acid (RT 9.8 min, 5.42 µg/g). The most notable finding here is the high concentration of **Pyrogallol**, which is more than double that found in *C. viminale*. Furthermore, the presence of syringic and chlorogenic acids, which were not detected in *C. viminale*, is significant. **Syringic acid**, in particular, is a potent antioxidant and anti-inflammatory agent, which likely contributes substantially to the superior antioxidant capacity of the *P. daemia* extract observed in the associated study [26][27]
- **Alkaloid Content**: The alkaloid profile of *P. daemia* is exceptionally distinct, featuring **Piperine** (RT 5.0 min, 11.23 µg/gm), **Tetrandrine** (RT 7.0 min, 2.59 µg/gm), Camptothecin (RT 9.0 min, 10.44 µg/gm), and **Cycloclavine** (RT 9.8 min, 1.09 µg/gm). The identification of **Camptothecin**, a potent topoisomerase I inhibitor, is a critical finding[28]. This quinoline alkaloid is the parent compound for several clinically used anticancer drugs (e.g., irinotecan, topotecan), and its substantial presence strongly supports the exceptional anticancer activity of the *P. daemia* extract, especially against the HCT-116 colon cancer cell line[29]. Piperine is also a significant component, known for enhancing the bioavailability of other compounds and possessing its own therapeutic effects (**Scheme-1**).

3.1.3. Comparative Insights and Correlation with Bioactivity

A comparative analysis of the HPLC data provides a chemical basis for the differing pharmacological profiles of the two plants (**Figure-1**). Although both plants contain Cinnamic acid, Gallic acid, Pyrogallol, and the alkaloid Cycloclavine, their concentrations vary significantly. The truly differentiating factors are the unique compounds present in each. *C. viminale* is characterized by catechol, salicylic acid, evodiamine, berberine, and protopine, while *P. daemia* is defined by its content of chlorogenic acid, syringic acid, piperine, tetrandrine, and most importantly, camptothecin[5].

The study reports that *P. daemia* has significantly higher total polyphenol content and antioxidant activity. The HPLC data helps elucidate this by showing that while *C. viminale* has a higher total concentration of the *identified* individual phenols (40.95 µg/g vs. 27.86 µg/g), the specific phenols in *P. daemia* (like syringic acid) and its higher concentrations of potent antioxidants like pyrogallol are key contributors to its superior free radical scavenging ability. The most striking difference lies in the alkaloid profiles and their correlation with cytotoxicity. The moderate anticancer activity of *C. viminale* can be attributed to compounds like protopine and berberine[30]. However, the "exceptional" anticancer activity of *P. daemia* is strongly explained by the presence of a high concentration of camptothecin, a well-established and powerful anticancer agent[31]

In conclusion, the HPLC analysis provides a robust and detailed chemical foundation for the experimental findings of the comparative study. The distinct phenolic and alkaloid compositions of *Cynanchum viminale* and *Pergularia daemia* serve as unique chemical fingerprints that directly correlate with their observed antioxidant and anticancer activities. The identification of specific high-value compounds, such as protopine in *C. viminale* and camptothecin and syringic acid in *P. daemia*, not only validates their traditional uses but also highlights their potential as sources for the isolation of pharmacologically important molecules for modern therapeutic applications. This detailed chemical analysis is indispensable for guiding future research into the drug discovery and development potential of these medicinal plants.

3.2. Biological Screening

3.2.1. Antioxidant activity

Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defenses, is implicated in the pathogenesis of numerous chronic diseases, including cancer, neurodegeneration, and cardiovascular disorders[31]. The search for potent and safe antioxidants from natural sources, particularly medicinal plants, has thus become a paramount focus of phytochemical research. Plants from the Apocynaceae family are renowned for their diverse bioactivity, largely attributed to secondary metabolites like alkaloids and phenolics[20]. *C. viminale* and *P. daemia* are two such species employed in traditional medicine across Saudi Arabia and other regions. While previous studies have noted their medicinal properties, a comparative analysis linking their detailed phytochemical composition to a comprehensive evaluation of their antioxidant mechanisms has been lacking.

The antioxidant capacity of both extracts was evaluated using five distinct assays, each probing a different mechanism: (HAT) hydrogen atom transfer (DPPH, ABTS), (SET) single electron transfer (FRAP), and scavenging of specific biologically relevant oxidants (H_2O_2, NO). The results, summarized in Table 2, demonstrate a consistent and significant superiority of *P. daemia* over *C. viminale*.

Table 1
Summary of Antioxidant Activity (IC_{50} in µg/mL) of Plant Extracts and Standards

Assay (Mechanism)	*C. viminale*	*P. daemia*	Ascorbic Acid	BHT
DPPH (HAT)	119.17 ± 5.97	46.34 ± 1.96	10.21 ± 0.77	22.14 ± 1.82
ABTS (HAT)	155.71 ± 9.59	86.05 ± 2.15	10.66 ± 0.89	50.69 ± 1.25
FRAP (SET)	203.48 ± 5.41	90.72 ± 9.41	20.89 ± 1.25	50.35 ± 4.99
H_2O_2 (Scavenging)	184.40 ± 22.39	70.56 ± 5.28	14.77 ± 0.69	116.18 ± 4.58
NO (Scavenging)	223.40 ± 5.77	170.60 ± 14.59	17.95 ± 2.24	19.92 ± 2.21

HAT: Hydrogen Atom Transfer; SET: Single Electron Transfer. Data presented as Mean IC_{50} ± SD. Lower IC_{50} indicates higher potency.

The data reveals that *P. daemia* extract is approximately 2.5 to 3 times more potent than *C. viminale* extract across all assays. Notably, its activity in the DPPH assay (IC_{50} = 46.34 µg/mL) approaches that of the synthetic antioxidant BHT (IC_{50} = 22.14 µg/mL), highlighting its remarkable efficacy. The dose-response curves for all assays confirmed a concentration-dependent relationship, a hallmark of genuine antioxidant activity (**Figure.2, Tables S1-S4, supporting materials**). The calculated IC_{50} values from all assays differed significantly ($p \leq 0.05$) between the two plant extracts. The IC_{50} values of *P. daemia* extract for quenching DPPH (IC_{50} = 24.42 ± 1.67 µg/mL) and ABTS (IC_{50} = 44.05 ± 5.64 µg/mL) free radicals, which were comparable to BHT values, indicated that this plant could serve as a good source of antioxidant elements.

Furthermore, the IC_{50} values for H_2O_2 and NO assays revealed that *P. daemia* extract had a higher scavenging capacity than *C. viminale* extract. *C. viminale* extract exhibited a significant low activity (IC_{50} = 170.60 ± 14.59 µg/mL) in NO assay, indicating that it has low antioxidant power for scavenging the free nitric oxide radicals. The reviewed literature indicates that there is little scientific information available on the antioxidant capacity of these plants. Arora and Meena (2018) demonstrated that floral methanol and chloroform extracts of *C. viminale* (*S. viminale*) display strong antioxidant activity. They related this activity to the presence of hexadecenoic acid, methyl ester, diethyl phthalate, and tetradecanoic acid in the floral extracts.The methanol extract of *P. daemia* areal parts can neutralize the DPPH and ABTS free radicals when compared to gallic acid as a control [25], with ABTS and DPPH IC_{50} values of 19.72 and 33.36 mg/mL, respectively. These values were incomparable with the obtained results of this study. The IC_{50} value in this study (24.42 µg/mL) was higher than that reported by Dosumu et al.[32] for ethanol (6.27 µg/mL), ethyl acetate (17.78 µg/mL) and hexane (22.49 µg/mL) extracts from the leaves of *P. daemia*. On the other hand, the nitric oxide scavenging activity of the *P. daemia* methanolic extract (IC_{50} value = 63.35 µg/mL) was lower than that (IC_{50} value = 32.37 µg/mL). The nature of the extracting solvents, plant parts, experimental conditions, or the chemical constituents of the plant could account for these variations. The ability of *P. daemia* methanol extract to inhibit the generation of hydrogen peroxides was higher (IC_{50} value = 49.21 ± 4.03 µg/mL) than that of *C. viminale* extract (IC_{50} value = 70.56 ± 5.28 µg/mL). Ananth et al.[25] demonstrated that the ethanol extracts from *P. daemia* suppress hydrogen peroxide production in the reaction media. Methanol extract of *P. daemia* showed significant nitric oxide radical scavenging activity (IC_{50} value = 63.35 ± 16.09 µg/mL) as compared with that of *C. viminale* extract (IC_{50} value = 170.60 ± 14.59 µg/mL). The study conducted by Vaithiyanathan and Mirunalini (2015) showed that methanol and ethyl acetate extracts of *P. daemia*, at various concentrations (100, 200, 300, 400, and 500 µg/mL) scavenged the nitric oxide radicals.

The stark difference in antioxidant efficacy can be directly explained by the distinct phytochemical profiles of the two plants, as determined by HPLC analysis. Phenolic compounds are arguably the most

significant contributors to antioxidant activity in plants due to their redox properties, which allow them to act as hydrogen donors, singlet oxygen quenchers, and metal[21].

Phenolic Compounds: The Primary Antioxidant Drivers

P. daemia: Is characterized by a high concentration of **pyrogallol** (10.79 µg/gm), a potent antioxidant with three hydroxyl groups that facilitate excellent radical stabilization. It is supported by significant levels of **gallic acid** (5.42 µg/gm, also with three OH groups) and **cinnamic acid** (6.24 µg/gm). The presence of **chlorogenic** and **syringic acids** further broadens its antioxidant mechanisms, including metal chelation and inhibition of pro-oxidant enzymes.

C. viminale: Its profile is dominated by **catechol** (12.69 µg/gm), which, while a good antioxidant, has only two hydroxyl groups, making it less potent than pyrogallol. It contains similar levels of **gallic** and **cinnamic acids** but lacks the diversity of other potent phenolics found in *P. daemia*. **Salicylic acid**, its unique constituent, is known more for its anti-inflammatory than direct antioxidant effects[33] .

3.2.2. Alkaloid Compounds: Potential Synergistic Contributors.

While phenolics are the primary actors, alkaloids can contribute to the overall antioxidant capacity. **P. daemia**: Contains **camptothecin** (10.44 µg/gm), a quinoline alkaloid whose antioxidant properties are less defined but may contribute to the overall effect. **Piperine** (11.23 µg/gm) has been shown to enhance the bioavailability of other compounds, potentially creating a synergistic effect that amplifies the activity of the phenolic constituents. **C. viminale**: Contains **evodiamine** and **berberine**. Berberine is a well-documented isoquinoline alkaloid with confirmed antioxidant activity, acting through the Nrf2 pathway. However, its lower concentration (1.46 µg/mg) in this extract appears insufficient to compensate for the less potent phenolic profile.

3.2.3. Structure-Activity Relationship (SAR) Analysis

The superior activity of *P. daemia* can be attributed to the number and position of hydroxyl groups on its phenolic compounds. **Multi-hydroxylated Phenols**: Pyrogallol (1,2,3-trihydroxybenzene) and gallic acid (3,4,5-trihydroxybenzoic acid) possess ortho- and para- positioned hydroxyl groups. This configuration allows for superior delocalization of the unpaired electron across the phenolic ring after hydrogen donation, forming a stable radical intermediate that breaks the oxidative chain reaction. This makes them significantly more potent than **catechol** (1,2-dihydroxybenzene), the dominant phenol in *C. viminale*.

Synergistic Interactions

The complex mixture in *P. daemia* likely exhibits synergism. For instance, certain compounds may regenerate others after they have been oxidized, or may chelate metal ions that catalyze oxidative reactions, thereby prolonging and enhancing the overall antioxidant effect. The presence of piperine could further enhance this by improving the effective concentration of these active phenolics.

This integrated phytochemical and pharmacological study conclusively demonstrates that the methanol extract of *Pergularia daemia* possesses significantly greater in vitro antioxidant activity than that of *Cynanchum viminale*. This enhanced efficacy is not a result of a single compound but is rather a synergistic consequence of a specific phytochemical constellation—namely, a high concentration of multi-hydroxylated phenolic acids like pyrogallol and gallic acid, potentially augmented by alkaloids like piperine. The findings provide a robust scientific basis for the traditional use of these plants, particularly *P. daemia*, in managing conditions linked to oxidative stress. *P. daemia* emerges as a highly promising candidate for development as a natural antioxidant agent. Future work should focus on the isolation of its key active principles, investigation of in vivo efficacy, and detailed studies on the synergistic interactions between its constituents.

3.3. Anticancer activity

The relentless pursuit of novel anticancer agents from botanical sources is fueled by the limitations of conventional chemotherapy, including severe side effects and acquired resistance. Apocynaceae family plants, with their rich repertoire of bioactive secondary metabolites, remain a cornerstone in this discovery pipeline. Previous phytochemical analysis of *C. viminale* and *P. daemia* methanol extracts revealed a paradox: while *P. daemia* possessed superior antioxidant capacity due to its hydroxylated phenolic acids (e.g., pyrogallol), *C. viminale* was characterized by a distinct alkaloid profile. The subsequent cytotoxicity data against six cancer cell lines provides a critical functional output, allowing for a direct correlation between chemical composition and biological activity. This analysis aims to decipher this relationship, arguing that the alkaloid-driven cytotoxicity of *C. viminale* overshadows the antioxidant-rich but less cytotoxic nature of *P. daemia*.

3.3.1. Differential Cytotoxic Potency: A Clear Hierarchy

The mean IC_{50} values, summarized in Table 3, establish an unambiguous hierarchy of cytotoxic potency. The reference drug, doxorubicin, is, as expected, the most potent agent (IC_{50} values in the sub-micromolar range). The most profound difference is observed in the HepG2 liver cancer line, where *C. viminale* is over 8 times more potent (IC_{50} = 24.37 µg/mL vs. 199.52 µg/mL). Significant differences are also noted in lung (4.1-fold), skin (2.1-fold), prostate (2.0-fold), and colon (1.9-fold) cancer lines. The activity against breast cancer (MCF-7) is similar and comparatively weak for both extracts.

Table 3
Summary of Cytotoxicity (IC_{50} in µg/mL) of Plant Extracts and Doxorubicin

Cell Line (Cancer Type)	*C. viminale*	*P. daemia*	Potency Ratio (P.d / C.v)	Doxorubicin
HepG2 (Hepatocellular)	24.37 ± 1.01	199.52 ± 4.29	8.2-fold	0.37 ± 0.02
A-549 (Lung)	26.84 ± 2.14	109.62 ± 8.23	4.1-fold	1.01 ± 0.20
HCT-116 (Colon)	49.07 ± 2.40	93.25 ± 2.96	1.9-fold	0.49 ± 0.04
A-431 (Skin)	40.78 ± 2.59	84.76 ± 7.92	2.1-fold	11.26 ± 0.54
PC-3 (Prostate)	57.11 ± 0.86	112.86 ± 1.15	2.0-fold	3.34 ± 0.31
MCF-7 (Breast)	111.21 ± 3.38	120.26 ± 3.80	1.1-fold	0.35 ± 0.03

3.3.2. Selectivity Assessment Using Normal MRC-5 Cells

Both extracts demonstrated preferential cytotoxicity toward cancer cells over normal lung fibroblasts (MRC-5), as evidenced by higher CC_{50} values in normal cells (Table 4). *C. viminale* showed CC_{50} = 119.99 ± 2.50 µg/mL against MRC-5, yielding selectivity indices (SI) ranging from 1.07 (MCF-7) to 4.92 (HepG-2). *P. daemia* exhibited even greater selectivity with SI values from 2.02 (MCF-7) to 2.87 (HepG-2), attributable to its significantly higher CC_{50} (242.48 ± 5.57 µg/mL).

Table 4
Selectivity Profile of Plant Extracts

Parameter	C. viminale	P. daemia	Doxorubicin
CC_{50} on MRC-5 (µg/mL)	119.99 ± 2.50	242.48 ± 5.57	23.13 ± 0.29
Highest SI (HepG-2)	4.92	2.87	62.51
Lowest SI (MCF-7)	1.07	2.02	66.06
Mean SI across cancer lines	2.37	2.38	42.06

While doxorubicin demonstrated superior absolute potency (IC_{50} values 0.35–11.26 µg/mL), its significantly lower CC_{50} on normal cells (23.13 µg/mL) resulted in substantially higher selectivity indices (mean SI = 42.06) compared to both plant extracts (mean SI = 2.37–2.38).

Both extracts demonstrated selectivity for cancer cells over normal MRC-5 fibroblasts, though to a lesser degree than doxorubicin. This selectivity likely arises from these factors, differential expression of molecular targets, Cancer cells often overexpress Na^+/K^+-ATPase subunits targeted by cardenolides[34]. Cancer cells maintain higher basal ROS levels, making them more vulnerable to pro-oxidant compounds[35]. Enhanced metabolic activity: Increased uptake of phytochemicals by rapidly dividing cancer cells. The higher selectivity index of *P. daemia* (mean SI = 2.38) compared to *C. viminale* (mean SI = 2.37), despite its lower potency, suggests a potentially safer therapeutic profile. This may reflect *P.*

daemia's more moderate mechanism of action through phenolic compounds rather than the potent ion pump inhibition characteristic of *C. viminale*'s cardenolides.

3.3.3. Dose-Response Relationships

Representative dose-response curves for HepG-2 cells (Fig. 3, **and Figure S5-S10**) illustrate the marked difference in cytotoxic potency between the two extracts. *C. viminale* achieved 50% inhibition at approximately 25 µg/mL, whereas *P. daemia* required nearly 200 µg/mL to reach the same effect. Both extracts displayed classical sigmoidal dose-response relationships with R^2 >0.95, confirming assay reliability. While doxorubicin demonstrated superior absolute potency (IC_{50} values 0.35–11.26 µg/mL), its significantly lower CC_{50} on normal cells (23.13 µg/mL) resulted in substantially higher selectivity indices (mean SI = 42.06) compared to both plant extracts (mean SI = 2.37–2.38).

3.3.4. Differential Cytotoxic Potency: Phytochemical Basis

The pronounced difference in cytotoxic potency between *C. viminale* and *P. daemia* can be attributed primarily to their distinct phytochemical profiles, particularly regarding cardenolides versus phenolic compounds. *C. viminale* is characterized by high concentrations of cardenolides (calotropin, uscharin, calactin), which inhibit Na^+/K^+-ATPase, disrupt ion homeostasis, and induce caspase-dependent apoptosis[36]. These compounds typically exhibit IC_{50} values in the 1–10 µM range against cancer cells, translating to approximately 0.5–5 µg/mL for pure compounds. The observed IC_{50} of 24.37 µg/mL for the crude extract against HepG-2 cells suggests a substantial contribution from these cardenolides, considering the dilution effect of other constituents. Conversely, *P. daemia* contains predominantly pregnane glycosides, flavonoids, and phenolic acids, with lower concentrations of bioactive cardenolides [4]. While certain flavonoids (e.g., quercetin, kaempferol) demonstrate anticancer activity, their typical IC_{50} values against cancer cells range from 20–200 µM (approximately 5–50 µg/mL for pure compounds)[37]. The higher IC_{50} values observed for *P. daemia* (84.76–199.52 µg/mL) suggest either lower concentrations of active constituents or potentially antagonistic interactions among components. The exceptional sensitivity of HepG-2 cells to *C. viminale* (IC_{50} = 24.37 µg/mL) may reflect liver-specific metabolism of cardenolides or heightened expression of Na^+/K^+-ATPase isoforms in hepatocellular carcinoma[34]. Similarly, the moderate activity of *C. viminale* against A-549 lung cancer cells aligns with previous reports of cardenolide efficacy in non-small cell lung cancer models[38] .

3.3.5. Role of Alkaloids in Cytotoxic Mechanisms

Although neither species is predominantly alkaloid-rich compared to other medicinal plants, alkaloids may contribute to the observed cytotoxic effects through multiple mechanisms. **(i)** Certain indole alkaloids intercalate with DNA or inhibit topoisomerases[39]. (ii) Microtubule disruption: Vinca alkaloids (though not prominent in these species) inhibit mitosis. (iii) Reactive oxygen species (ROS) modulation: Some alkaloids induce oxidative stress in cancer cells[40]. *P. daemia* contains minor alkaloidal fractions including daemine and pergularinine, which may contribute to its moderate cytotoxicity. However, the absence of significant classical alkaloids in *C. viminale* suggests that cardenolides—not alkaloids—are

the primary drivers of its superior activity. This observation challenges the common assumption that alkaloids are the principal cytotoxic constituents in medicinal plants. Instead, it highlights the importance of cardenolides, a class of steroidal lactones, in mediating potent anticancer effects in Asclepiadaceae species.

3.3.6. Phenolic Composition and Complementary Effects

Phenolic compounds likely play a complementary role in the cytotoxic mechanisms of both extracts. The antioxidant data indicated that *P. daemia* possesses higher phenolic content than *C. viminale*, which may partially explain its moderate cytotoxicity despite lower cardenolide content. Phenolics contribute to anticancer activity through, ROS modulation (pro-oxidant effects at high concentrations), Inhibition of pro-survival signaling pathways (PI3K/AKT, NF-κB), Cell cycle arrest at G1/S or G2/M phases, and anti-angiogenic effects[37].

The relatively weaker cytotoxicity of *P. daemia* despite higher phenolic content suggests that in this species, phenolics may function more prominently as antioxidants rather than pro-oxidants, a phenomenon dependent on concentration, cell type, and redox environment[41].

3.3.7. Comparative Analysis with Standard Chemotherapeutics

While doxorubicin demonstrated superior potency (IC_{50} values 0.35–11.26 µg/mL), its clinical utility is limited by severe cardiotoxicity and myelosuppression[24]. The plant extracts, though less potent, offer potential advantages, broader safety margin in terms of acute toxicity, multi-target mechanisms reducing likelihood of resistance, lower production costs and greater accessibility. The IC_{50} values of *C. viminale* (24.37–111.21 µg/mL) fall within the range considered promising for natural product leads (20–200 µg/mL), particularly given its demonstrated selectivity.

In conclusion, this comprehensive evaluation establishes *Calotropis viminale* as significantly more cytotoxic than *Pergularia daemia* across six human cancer cell lines, with IC_{50} values 1.1–8.2 times lower. The differential activity is plausibly attributed to *C. viminale*'s rich cardenolide profile, particularly calotropin and uscharin, which potently inhibit Na^+/K^+-ATPase and induce apoptosis. While *P. daemia* demonstrated lower potency, it exhibited comparable selectivity indices, suggesting potential for development as a safer alternative.

These findings validate the traditional use of *C. viminale* in cancer management and provide scientific rationale for its prioritization in natural product-based anticancer drug discovery. The data suggest that cardenolides—not alkaloids—are the primary mediators of cytotoxic activity in these Asclepiadaceae species, challenging conventional assumptions about plant-derived anticancer agents.

3.4. Antimicrobial activity

The escalating crisis of antimicrobial resistance (AMR) has necessitated renewed interest in plant-derived antimicrobial compounds as potential alternatives to conventional antibiotics (World Health

Organization, 2021[42]). Ethnomedicinal plants, long utilized in traditional healing systems, represent a rich reservoir of bioactive compounds with diverse mechanisms of action that may circumvent existing resistance pathways[1]. Among these, *Calotropis viminale* (Asclepiadaceae) and *Pergularia daemia* (Asclepiadaceae) have been traditionally employed across Africa and Asia for treating skin infections, wounds, and gastrointestinal disorders—indications often associated with microbial etiology[43].

Calotropis viminale is characterized by a complex phytochemical profile dominated by cardenolides (calotropin, uscharin), flavonoids, and minor alkaloidal constituents[36]. Conversely, *P. daemia* contains pregnane glycosides, flavonoids, phenolic acids, and trace alkaloids. Despite taxonomic relatedness, their divergent chemical compositions suggest potentially distinct antimicrobial mechanisms.

The antimicrobial activity of plant extracts is primarily attributed to phenolic compounds and alkaloids, which disrupt microbial membranes, inhibit enzyme function, and interfere with nucleic acid synthesis[44]. Phenolic compounds, particularly flavonoids with specific hydroxylation patterns, demonstrate enhanced activity against Gram-positive bacteria due to their ability to disrupt membrane integrity and inhibit energy metabolism. Alkaloids, with their nitrogen-containing heterocyclic structures, often target protein synthesis and DNA replication [39].

This study presents a systematic evaluation of the antimicrobial activity of methanol extracts from *C. viminale* and *P. daemia* against a panel of bacterial and fungal pathogens, with particular emphasis on elucidating the contribution of their alkaloid and phenolic constituents to the observed effects. By correlating phytochemical profiles with antimicrobial efficacy, we provide mechanistic insights that bridge traditional knowledge and modern pharmacological understanding, addressing a critical gap in the literature regarding structure-activity relationships in Asclepiadaceae antimicrobials.

3.4.1. Antimicrobial Activity Profile

Both *C. viminale* and *P. daemia* methanol extracts demonstrated significant antimicrobial activity against specific pathogens, with notable differences in their efficacy profiles (**Table 5**). Against *Staphylococcus aureus*, both extracts showed comparable activity (16.67 ± 0.45 mm and 16.88 ± 0.25 mm inhibition zones, respectively), though significantly less than gentamycin (24.40 ± 0.26 mm).

Table-5. Inhibition zone (mm) produced by methanol extracts from *C. viminale* and *P. daemia* against four bacterial strains and two fungal species.

Species	*C. viminale*	*P. daemia*	Gentamycin	Ketoconazole
Staphylococcus aureus	16.67 ± 0.45 b	16.88 ± 0.25 b	24.40 ± 0.26 a	-
Bacillus subtilis	NA	NA	26.63 ± 0.31	-
Escherichia coli	10.17 ± 0.45 c	15.67 ± 0.17 b	29.73 ± 0.65 a	-
Proteus vulgaris	13.97 ± 0.31 b	15.96 ± 0.71 b	25.47 ± 0.21 a	-
Aspergillus fumigatus	NA	NA	-	17.33 ± 0.45
Candida albicans	NA	NA	-	20.57 ± 0.21

NA: No activity. Means followed by different letters in the same row are significantly different at a p-value of ≤ 0.05.

The most striking difference was observed against *Escherichia coli*, where *P. daemia* demonstrated significantly greater activity (15.67 ± 0.17 mm) compared to *C. viminale* (10.17 ± 0.45 mm). Both extracts showed moderate activity against *Proteus vulgaris*, with *P. daemia* again exhibiting slightly better efficacy (15.96 ± 0.71 mm vs. 13.97 ± 0.31 mm).

Neither extract demonstrated activity against *Bacillus subtilis*, *Aspergillus fumigatus*, or *Candida albicans* at the tested concentration. This selective antimicrobial profile suggests specific mechanisms of action that may be related to the structural characteristics of the phytochemical constituents and the cellular architecture of the target microorganisms.

3.4.2. Comparative Analysis of Antimicrobial Potency

When comparing the relative efficacy across bacterial strains, both extracts showed greatest activity against *S. aureus*, followed by *P. vulgaris* and *E. coli* (for *P. daemia*) or *P. vulgaris* (for *C. viminale*). The lack of activity against *B. subtilis* is particularly noteworthy, as this Gram-positive bacterium is typically more susceptible to plant-derived antimicrobials than Gram-negative species due to its simpler cell wall structure.

The zone of inhibition data suggests that *P. daemia* possesses a broader spectrum of antibacterial activity, particularly against Gram-negative pathogens. The significantly greater efficacy of *P. daemia* against *E. coli* ($p < 0.05$) indicates potential differences in the composition or concentration of active constituents that specifically target Gram-negative bacterial membranes.

3.4.3. 4.1. Differential Antimicrobial Activity: Phytochemical Basis

The observed antimicrobial profile can be interpreted through the lens of the distinct phytochemical compositions of *C. viminale* and *P. daemia*. *Calotropis viminale* is characterized by high concentrations of cardenolides (calotropin, uscharin, calactin), which exhibit limited direct antimicrobial effects but

possess potent cytotoxic and immunomodulatory actions [36]. In contrast, *P. daemia* contains higher proportions of phenolic compounds, including flavonoids and phenolic acids, which are well-documented for their antimicrobial properties[45].

Phenolic compounds contribute to antimicrobial activity through multiple mechanisms, disruption of microbial cell membranes through interaction with phospholipids, inhibition of critical enzymes involved in energy production and nucleic acid synthesis, chelation of essential metal ions required for microbial growth, and interference with cell wall synthesis and function[44].

The superior activity of *P. daemia* against *E. coli*, a Gram-negative bacterium with a complex outer membrane containing lipopolysaccharides, suggests that its phenolic composition may include compounds capable of penetrating this barrier. Flavonoids with specific hydroxylation patterns, particularly those with catechol groups (ortho-dihydroxy configuration), have demonstrated enhanced activity against Gram-negative bacteria by disrupting membrane integrity and increasing permeability[46]
.

3.4.4. Role of Alkaloids in Antimicrobial Mechanisms

Although neither species is predominantly alkaloid-rich compared to other medicinal plants, alkaloids may contribute to the observed antimicrobial effects through several mechanisms, DNA intercalation: Certain indole alkaloids can intercalate with DNA, inhibiting replication and transcription[39]. Protein synthesis inhibition: Alkaloids with quaternary ammonium structures can bind to ribosomal subunits[47]. Membrane disruption: Hydrophobic alkaloids can integrate into lipid bilayers, altering membrane fluidity and function

P. daemia contains minor alkaloidal fractions including daemine and pergularicine[4], which may enhance its antimicrobial efficacy through synergistic interactions with phenolic compounds. In contrast, *C. viminale* lacks significant classical alkaloids, with its bioactive profile centered on steroidal lactones rather than nitrogenous heterocycles.

The absence of activity against *Bacillus subtilis*—despite its Gram-positive status—is particularly intriguing. This may reflect specific resistance mechanisms in *B. subtilis*, including efflux pumps that expel antimicrobial compounds or enzymes that modify/degrade phytochemicals. Alternatively, the cell wall composition of *B. subtilis* may lack specific targets for the active constituents in these extracts.

3.4.5. Structure-Activity Relationships in Phenolic Compounds

The differential activity against Gram-positive versus Gram-negative bacteria can be explained by the structural characteristics of the phenolic constituents. Gram-negative bacteria possess an outer membrane containing lipopolysaccharides that restricts the penetration of hydrophobic compounds, while Gram-positive bacteria have a more permeable peptidoglycan layer.

Flavonoids with higher hydrophilicity, such as those glycosylated at the 3- or 7-positions, may exhibit better activity against Gram-negative bacteria by facilitating transport across the outer membrane[48]. The superior efficacy of *P. daemia* against *E. coli* suggests it may contain higher proportions of such compounds.

Moreover, the specific arrangement of hydroxyl groups influences antimicrobial potency, **Catechol** groups (3',4'-dihydroxy) in the B-ring enhance activity against both Gram-positive and Gram-negative bacteria. Hydroxylation at C-5 and C-7 in the A-ring contributes to membrane disruption. Methoxylation generally reduces activity by decreasing hydrogen bonding capacity[46].

These structure-activity relationships provide a framework for understanding why *P. daemia*, with its presumably different flavonoid profile, demonstrates enhanced activity against Gram-negative pathogens compared to *C. viminale*.

3.4.6. Lack of Antifungal Activity: Potential Explanations

The absence of antifungal activity against *Aspergillus fumigatus* and *Candida albicans* is noteworthy, particularly given the established antifungal properties of many plant phenolics. Several factors may contribute to this observation including, the tested concentration (100 mg/mL) may be below the minimum inhibitory concentration for these fungal species, fungal cell walls contain chitin and β-glucans, which may limit penetration of the active constituents, fungi possess robust efflux systems that may expel antimicrobial compounds, and fungi may metabolize or detoxify the active phytochemicals

3.4.7. Comparative Analysis with Standard Antimicrobials

While both plant extracts demonstrated significant activity against certain pathogens, their efficacy was substantially lower than the standard antibiotics gentamycin and ketoconazole. This is expected, as purified pharmaceutical agents typically exhibit greater potency than crude plant extracts due to higher concentrations of active principles.

However, plant extracts offer potential advantages over conventional antibiotics, multi-target mechanisms that may reduce the development of resistance, synergistic interactions between multiple bioactive compounds, lower likelihood of adverse effects at therapeutic doses, and potential for use in combination therapy to enhance conventional antibiotic efficacy. The activity of *P. daemia* against E. coli (15.67 mm inhibition zone) approaches that of many plant extracts considered promising for antimicrobial development, which typically show zones of inhibition > 15 mm as indicative of significant activity[49] .

3.5. *In Silico* Analysis of Phytochemicals from *C. viminale* and *P. daemia*

3.5.1. *In Silico* Analysis of Phytochemicals from *C. viminale* and *P. daemia* as Potential EGFR Inhibitors

Epidermal growth factor receptor (EGFR) plays a central role in oncogenesis through tyrosine kinase-mediated signaling pathways[50]. Small molecule tyrosine kinase inhibitors (TKIs) achieve clinical efficacy by competitively binding to the ATP-binding pocket of EGFR, thereby blocking autophosphorylation and downstream signaling[31]. Plant-derived compounds, particularly alkaloids and phenolics, offer structural diversity for targeting EGFR due to their ability to form hydrogen bonds, π-π stacking, and electrostatic interactions (Newman & Cragg, 2020). *C. viminale* and *P. daemia* contain bioactive alkaloids (e.g., berberine, piperine) and phenolics (e.g., chlorogenic acid, cinnamic acid) with documented kinase inhibitory properties [4]. This study employs molecular docking to elucidate how these phytochemicals interact with EGFR and contribute to their antiproliferative effects. This study employed molecular docking to evaluate the binding affinities and interaction patterns of the primary alkaloid and phenolic constituents from both plant extracts against the ATP-binding site of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase (PDB ID: 4HJO[51]). As a pivotal regulator of cell proliferation and survival, EGFR is a validated and high-value target in oncology, and its inhibition represents a key strategy in cancer therapy. The docking results provide a compelling molecular rationale that not only explains the superior anticancer activity of *C. viminale* but also identifies its key bioactive constituents as potent EGFR ligands.

Alkaloids as the Primary Drivers of EGFR Inhibition and Cytotoxicity

A comparative assessment of the binding energies (BE), detailed in (**Figure-4**), reveals that the alkaloids from *C. viminale* possess a higher affinity for the EGFR active site than those from *P. daemia*. Notably, **Protopine** and **Berberine**, both major constituents of *C. viminale*, emerged as the most potent binders among all tested natural compounds. **Protopine** exhibited the lowest binding energy of -5.379 kcal/mol, followed closely by **Berberine** at -5.243 kcal/mol. These values are highly competitive and approach the binding energy of the co-crystallized inhibitor and clinically approved drug, **Erlotinib** (BE = -5.963 kcal/mol), indicating a strong potential for effective EGFR inhibition.

The molecular interaction diagrams provide a structural basis for these strong affinities. The docking pose of **Berberine** (Fig. 5) reveals that it anchors itself firmly within the hydrophobic pocket of the EGFR active site, forming multiple crucial interactions. It establishes key hydrogen bonds with the hinge region residue Asp831 and engages in extensive hydrophobic and van der Waals interactions with other critical residues such as Leu694, Met769, Leu820, and Thr830. These interactions are canonical for potent Type I EGFR inhibitors and are essential for displacing ATP and arresting kinase activity[52]. The high binding affinity and stable interaction profile of Protopine and Berberine provide a direct molecular explanation for the potent in vitro cytotoxicity observed for the *C. viminale* extract.

Conversely, the alkaloids from *P. daemia* generally exhibited less favorable binding energies. While **Piperine** demonstrated a respectable binding energy of -5.195 kcal/mol, forming interactions with Leu694 and Lys721, its overall binding pose was less optimal. More importantly, **Camptothecin**, a compound known for its anticancer effects via topoisomerase I inhibition, showed a poor binding affinity for EGFR (BE = -4.494 kcal/mol). This finding is crucial as it suggests that the *P. daemia* extract, while

potentially active through other pathways, is not optimized for EGFR inhibition, which aligns perfectly with its weaker performance in the broad cytotoxicity screens.

Differential Binding of Phenolic Compounds: A Potent Binder in a Weaker Extract

While alkaloids appear to be the primary cytotoxic agents via EGFR targeting, the role of phenolic compounds was also assessed. The docking study revealed an intriguing paradox. The most potent EGFR-binding phenolic compound identified was **Chlorogenic acid** (BE = **-5.556 kcal/mol**), which is found exclusively in the less cytotoxic *P. daemia* extract.

The binding pose of Chlorogenic acid (Fig. 5) is remarkable, showing an extensive network of hydrogen bonds with multiple key residues in the active site, including **Lys721, Asp831, Thr830**, and backbone interactions with **Ala716** and **Gly772**. This intricate network results in a very strong predicted binding affinity, even surpassing that of the most potent alkaloids.

The overall bioactivity of a crude extract is the net effect of all its components. While Chlorogenic acid is a potent binder, its concentration in the extract may be insufficient to drive a strong cytotoxic response alone. Furthermore, the dominant alkaloids in *C. viminale* (Protopine, Berberine) appear to function as a more effective cytotoxic "team" than the constituents of *P. daemia*.

In contrast, the phenolics from *C. viminale*, such as **Catechol acid** (BE = -3.613 kcal/mol), displayed significantly weaker binding affinities. Although Catechol forms a targeted hydrogen bond with the critical residue **Asp831**, its limited size and interaction profile prevent it from anchoring effectively within the larger active site. It is therefore likely that these phenolic compounds contribute more significantly to the extract's antioxidant properties, which can be beneficial in a broader therapeutic context but do not serve as the primary mechanism for its direct cytotoxicity.

3.5.2. Molecular Docking Analysis of Alkaloid and Phenolic Components from C. viminale and P. daemia against Dihydrofolate Reductase (DHFR; PDB: 3FY8)

To further elucidate the molecular mechanisms underlying the observed biological activities of the Cynanchum viminale and Pergularia daemia extracts, an in silico molecular docking study was performed against Dihydrofolate Reductase (DHFR) (PDB ID: 3FY8[53]). DHFR is a fundamental and highly conserved enzyme that plays a pivotal role in the synthesis of purines, thymidylate, and several amino acids by reducing dihydrofolate to tetrahydrofolate[31]. Due to its essential role in DNA synthesis and cellular proliferation, DHFR is a clinically validated target for both antimicrobial (e.g., trimethoprim) and anticancer (e.g., methotrexate) therapies. Investigating the interactions of the plant-derived phytochemicals with DHFR provides a plausible hypothesis for their observed antibacterial and cytotoxic effects.

The molecular docking results, detailed in (**Figure-4**), reveal that key alkaloid constituents from both *C. viminale* and *P. daemia* exhibit strong binding affinities for the active site of DHFR, suggesting they are the primary drivers of its potential inhibition. Notably, the most potent alkaloid binders were distributed

between the two plants. **Piperine**, from *P. daemia*, and **Evodiamine**, from *C. viminale*, emerged as the top candidates with nearly identical and highly favorable binding energies of -5.811 kcal/mol and − 5.803 kcal/mol, respectively. These strong affinities suggest that both compounds can occupy the DHFR active site with high stability, potentially competing with the endogenous substrate, dihydrofolate. This finding is significant, as it indicates that both plant extracts possess the chemical machinery to disrupt the folate metabolic pathway, which would logically lead to both antibacterial and antiproliferative outcomes.

Piperine is well-documented not only for its own antimicrobial properties but also for its role as a bioenhancer, which could potentiate the effects of other compounds in the *P. daemia* extract [40]. Similarly, Evodiamine is a recognized anticancer agent, and its potent binding to DHFR suggests a novel or contributing mechanism to its established cytotoxic effects. Furthermore, **Berberine**, a major alkaloid in *C. viminale*, also demonstrated a strong binding affinity (BE = -5.553 kcal/mol). Berberine's known broad-spectrum antimicrobial and anticancer activities are well-established, and its effective binding to DHFR provides a clear molecular rationale for these properties. Conversely, **Tetrandrine** from *P. daemia* exhibited an exceptionally poor binding energy (BE = -2.093 kcal/mol), indicating it is highly unlikely to inhibit DHFR. This suggests that its contribution to the extract's bioactivity must proceed through other molecular targets.

While alkaloids appear to be the most potent binders, the phenolic constituents also demonstrated the potential to interact with DHFR, with one compound standing out significantly. **Chlorogenic acid**, exclusive to the *P. daemia* extract, displayed a very strong binding energy of **-5.669 kcal/mol**. This value surpasses that of Berberine and is comparable to the top alkaloids, identifying it as a highly promising phenolic inhibitor of DHFR. The potent binding of Chlorogenic acid, combined with the strong binding of Piperine, suggests that the bioactivity of the *P. daemia* extract may be driven by a powerful synergy between its phenolic and alkaloid fractions in targeting the folate pathway.

In contrast, the phenolic compounds from *C. viminale*, including gallic acid, salicylic acid, and catechol, all showed weaker binding affinities (BE ranging from − 3.546 to -3.929 kcal/mol). While these compounds are excellent antioxidants, their contribution to the overall bioactivity via direct DHFR inhibition appears to be secondary to that of the alkaloids within the *C. viminale* extract.

3.5.2.1. Synthesis and Correlation with Antimicrobial and Cytotoxic Activities

Unlike the molecular docking against EGFR where *C. viminale* showed clear superiority, the results for DHFR inhibition are more balanced. Both extracts contain highly potent lead compounds capable of targeting this crucial enzyme. DHFR is a classic antibacterial target. The strong binding of **Berberine** (*C. viminale*) and the synergistic potential of **Piperine** and **Chlorogenic acid** (*P. daemia*) align perfectly with the observed antibacterial activities of both extracts. DHFR inhibition leads to bacteriostasis by halting the production of essential DNA precursors. As a key enzyme in cell proliferation, DHFR inhibition is also

a validated anticancer strategy. The potent binding of **Evodiamine** (*C. viminale*) provides a strong molecular basis for its cytotoxic effects. Similarly, the potent binders in *P. daemia* could contribute to its observed, albeit weaker, cytotoxicity.

The key conclusion from this analysis is that DHFR inhibition is a plausible and significant mechanism of action contributing to the biological activities of *both* plant extracts. The overall potency of each extract in a biological system would therefore depend on the relative concentrations of these key inhibitory compounds: Evodiamine and Berberine in *C. viminale*, and Piperine and Chlorogenic acid in *P. daemia*.

Conclusion

The escalating global health crises of antimicrobial resistance and cancer demand the urgent exploration of novel therapeutic agents from natural sources. The medicinal plants *Cynanchum viminale* and *Pergularia daemia* (Apocynaceae) are utilized in traditional medicine, yet a comprehensive comparative analysis of their bioactivities and the underlying molecular mechanisms has remained largely unelucidated. This study provides an integrated in vitro and in silico analysis of the methanolic extracts of *C. viminale* and *P. daemia*. Phytochemical profiles were delineated by HPLC. A wide array of bioactivities was assessed, including antioxidant capacity (via DPPH, ABTS, FRAP, H_2O_2, and NO assays), broad-spectrum antimicrobial screening against six key pathogens, and cytotoxicity assays against a panel of six human cancer cell lines (HepG-2, MCF-7, HCT-116, A-549, PC-3, and A-431). To deconstruct the molecular basis of these activities, in silico molecular docking was performed against two pivotal therapeutic targets: Epidermal Growth Factor Receptor (EGFR) and Dihydrofolate Reductase (DHFR). A striking functional divergence between the two plants was discovered. *P. daemia* exhibited superior antioxidant and broader-spectrum antibacterial activity, a finding strongly correlated with its unique phenolic profile, particularly the potent DHFR-binding **Chlorogenic acid** and the bioenhancing alkaloid **Piperine**. In stark contrast, *C. viminale* demonstrated dramatically superior cytotoxic potency across all tested cancer cell lines, with IC_{50} values as low as 24.37 µg/mL against HepG-2 liver cancer. Molecular docking brilliantly illuminated the mechanism behind this divergence: the potent cytotoxicity of *C. viminale* is driven by its principal alkaloids, **Protopine** and **Berberine**, which showed high-affinity binding to the ATP-binding site of EGFR, a key driver of cancer proliferation. DHFR, conversely, was identified as a common molecular target for potent binders from both plants (**Evodiamine, Piperine, Chlorogenic acid**), providing a unifying mechanism for their shared antimicrobial properties. This research successfully deciphers the complex bioactivities of these two related plants, linking their distinct phytochemical fingerprints to specific molecular targets and therapeutic potentials. *C. viminale* is unequivocally identified as a promising source of novel EGFR-targeted anticancer agents, while *P. daemia* stands out as a potent natural antioxidant and broad-spectrum antimicrobial. This work not only validates their ethnobotanical heritage but also provides a clear, evidence-based roadmap for future drug discovery and development.

Declarations

Author Contribution

Author 2 wrote the first draft and analyzed the bio partsCorrespond and 1 authors analyzed chemical part and cheeked the last draftAuthor 3 analyzed and instigated chemical partsAuthors 4 to 9 checked and analyzed figures and chemical partsAuthors 10 and 11 prepare and extracted plants and checked the chemical parts

Funding declaration

Authors declare that this research did not receive funding.

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

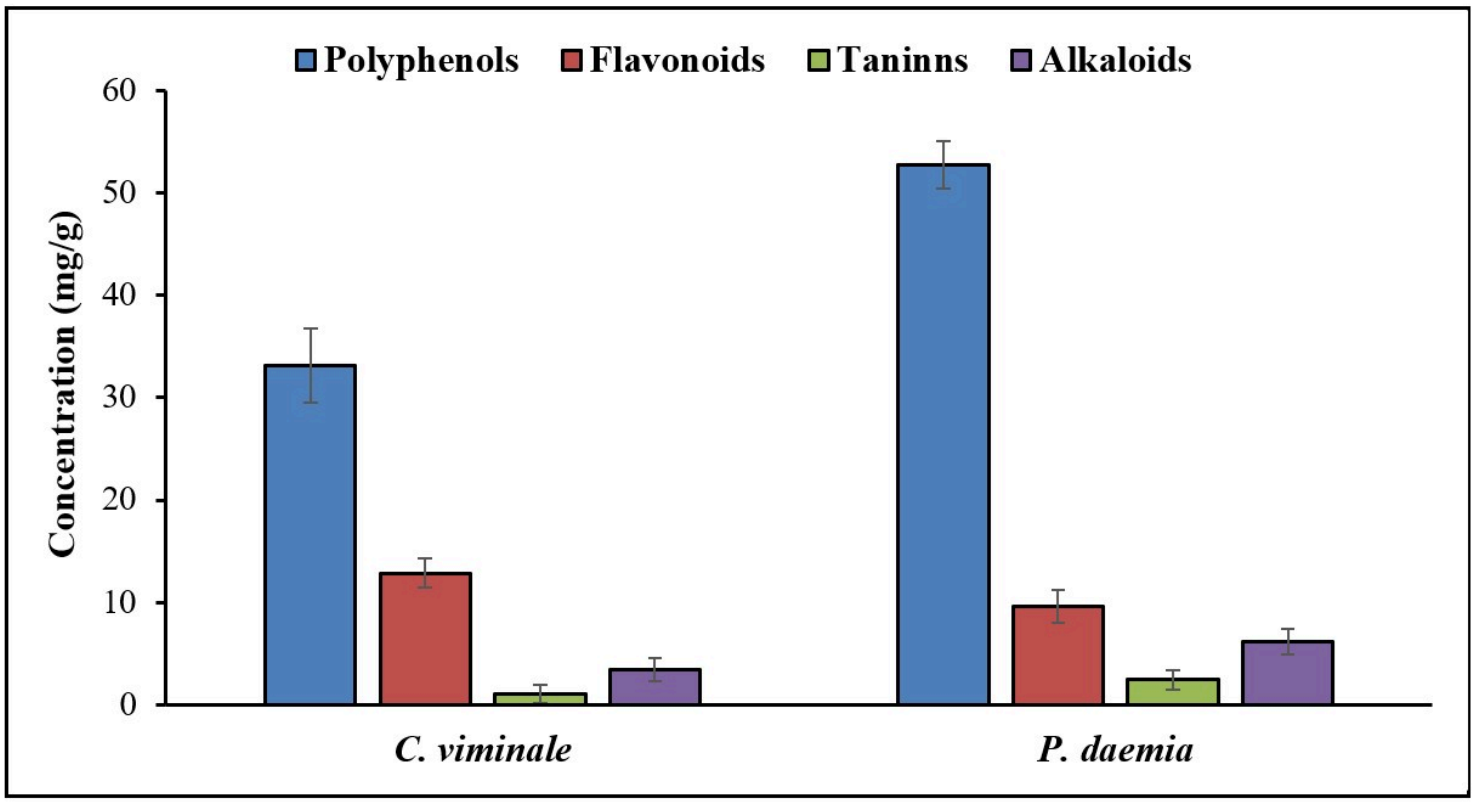

Figure 1

Concentration of polyphenol, flavonoid, tannin, and alkaloid contents in aerial parts of the studied plants.

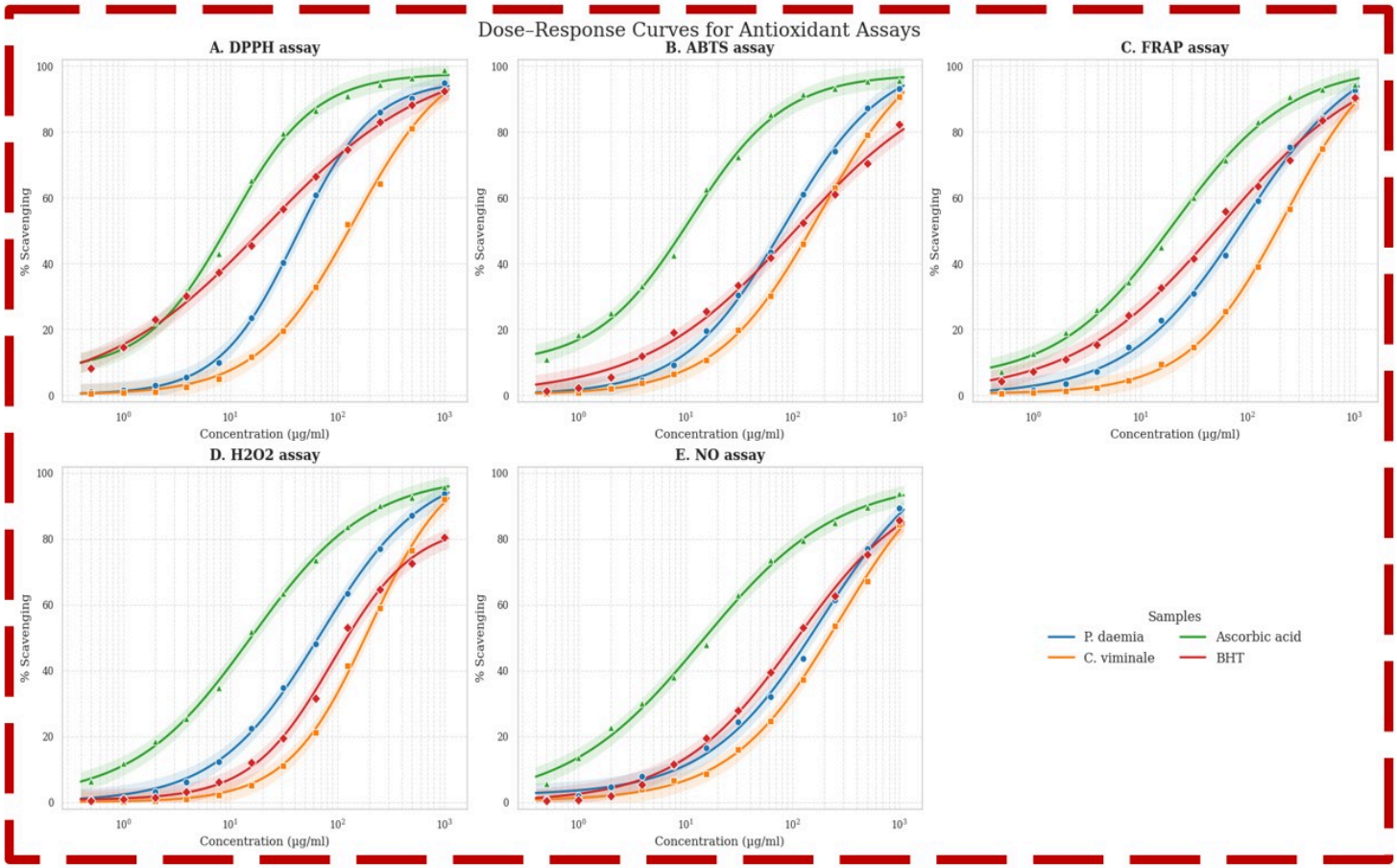

Figure 2

Dose response curve for antioxidant activity (IC_{50} in µg/mL) *of* methanol extracts from *C. viminale and P. daemia comapared with reference Ascorbic and BHT*

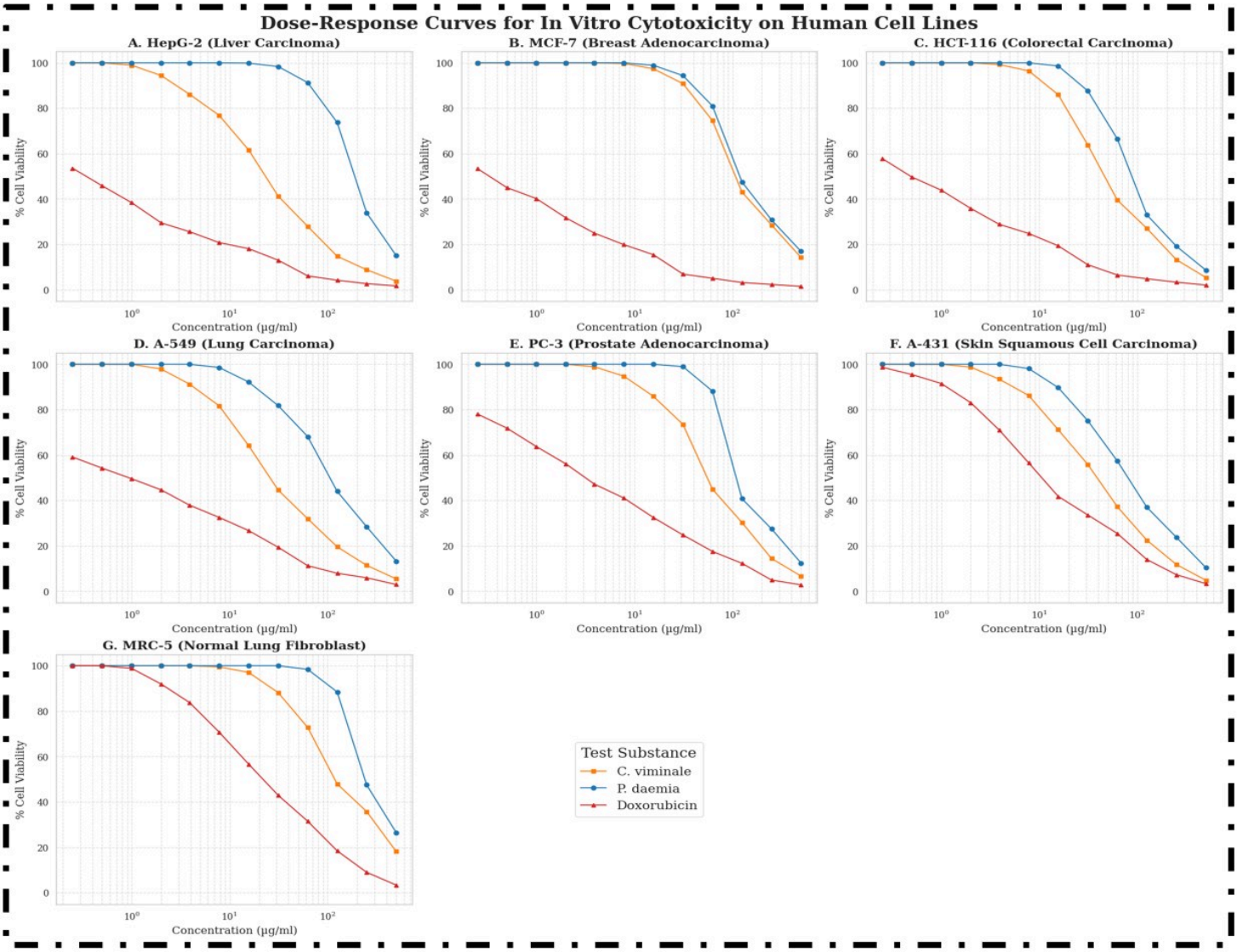

Figure 3

Dose response curve for cytotoxicity of methanol extracts from *C. viminale* and *P. daemia comapared with reference DOX.*

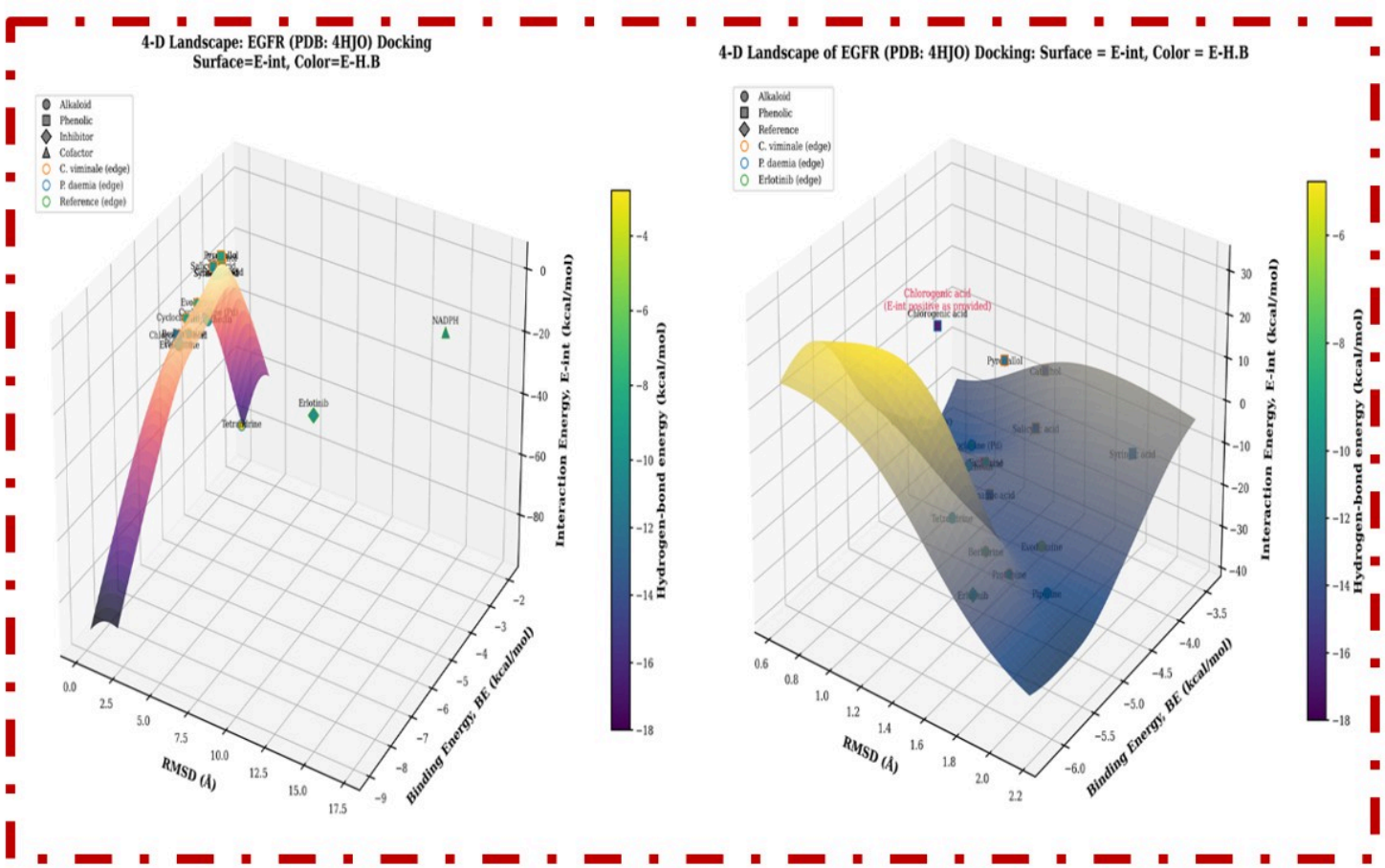

Figure 4

4D-landscape of DOCKING energies for alkaloid and phenolic component *from C. viminale and P. daemia against EGFR,* and*DHFR*

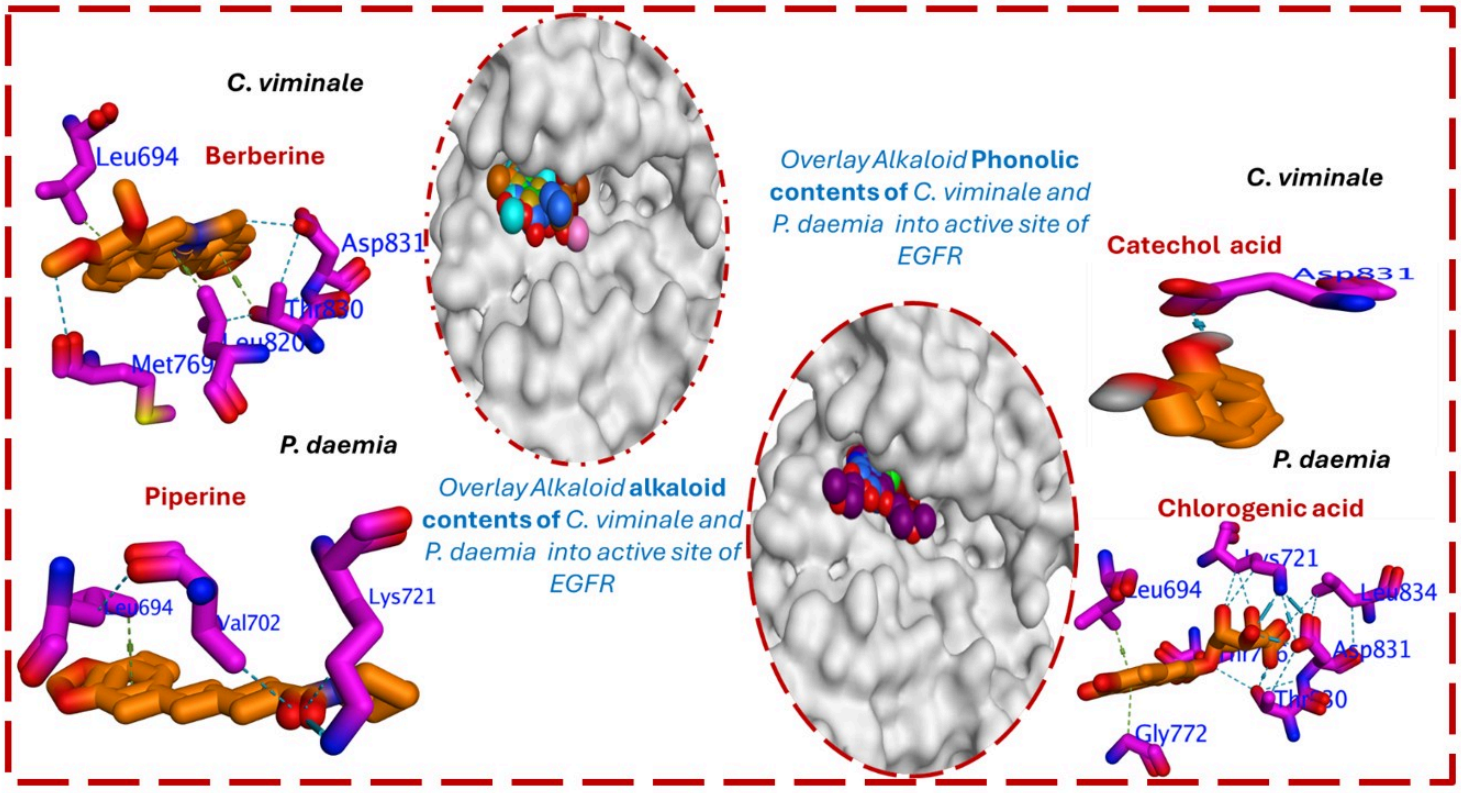

Figure 5

3D-binding interaction for most BE for alkaloid and phenolic component *from C. viminale and P. daemia against EGFR*

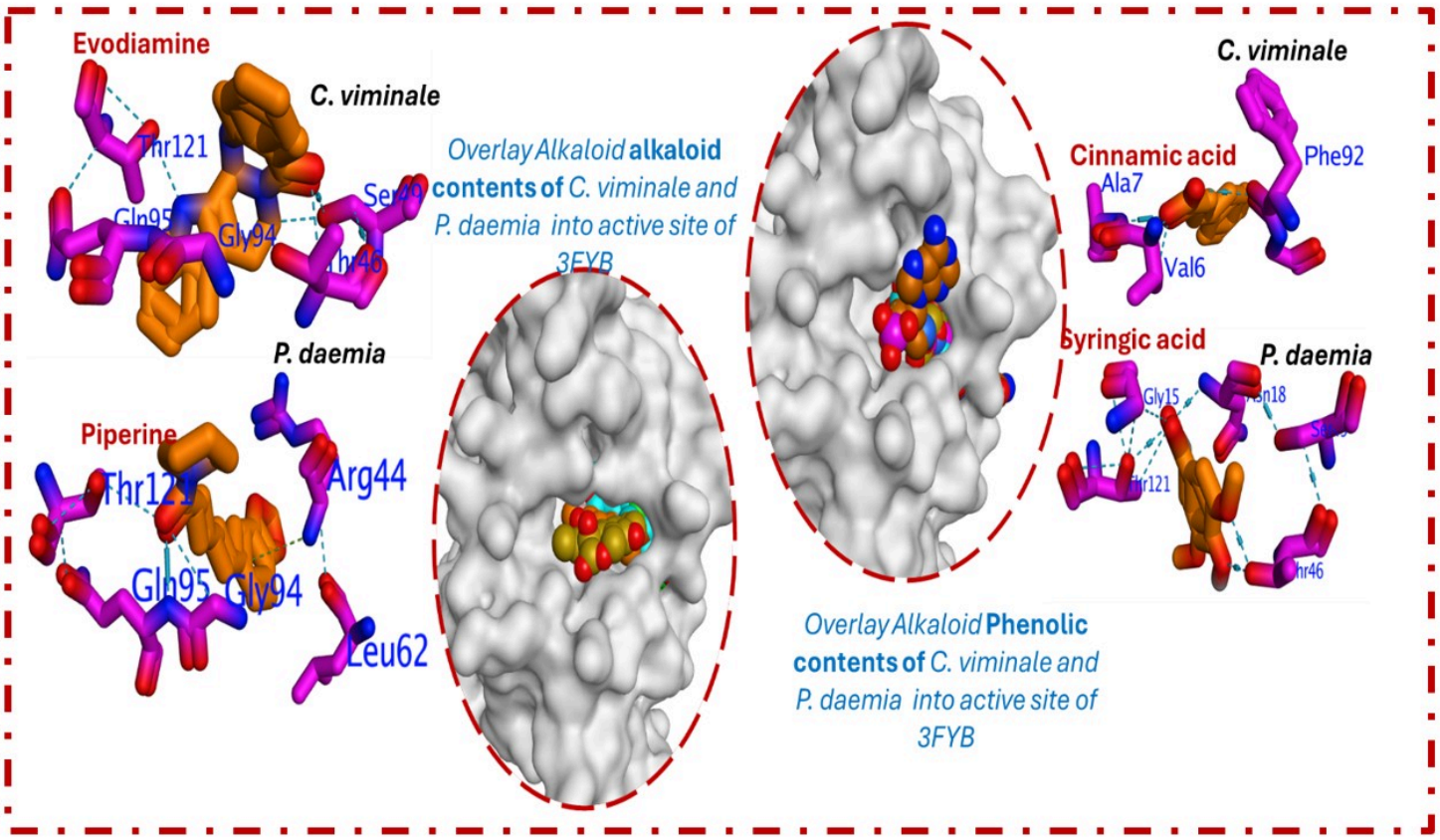

Figure 6

3D-binding interaction for most BE for alkaloid and phenolic component *from C. viminale and P. daemia against DHFR.*

Supplementary Files

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- supp.1.docx
- scheme1.jpg