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Evaluation of pharmacological properties and identification of biopotent compounds of the medicinal herb *Justicia simplex* D. Don

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Innovative research is going on in search of novel, safer, and more effective drugs that are affordable to the common man. The present study evaluates the wound healing, antibacterial, antifungal and anticancer efficacies of leaves of the folk medicinal herb *Justicia simplex*, identifies the bioactive compounds present in the extract and predicts the drug likeness and pharmacological activities of the compounds. The wound healing efficacy of the extract was evaluated by *in vitro* scratch assay, antimicrobial activities by agar well diffusion method, antioxidant activity by DPPH radical scavenging assay, cytotoxic activity by MTT assay, morphological studies were conducted using phase contrast microscopy, and cell apoptosis by acridine orange and ethidium bromide double staining method. FTIR and HRMS analyses were employed to identify the bioactive compounds present in the extract. Physicochemical characters and drug-likeness of the compounds were predicted employing the SWISSADME web tool and pharmacological activities by the PASS online software.The study proved the effectiveness of *J. simplex* against pathogenic bacteria, fungi, and its wound healing, anticancer and cell apoptotic properties. The bioactive compounds identified were ethyl 6,9,12-hexadecatrienoate, 17-octadecynoic acid, n-hexadecanoic acid and ethylene glycol 0,0-di(pivaloyl). Pa > 0.7 indicates that all the compounds are very likely to exhibit anti-eczematic activity and three compounds obey the Lipinski rule of drug likeness. While the study highlights *J. simplex* as a promising candidate for novel drug discovery, further research is needed to fully explore and validate its therapeutic potential.

Keywords: Anticancer, Antimicrobial, FTIR, HRMS, *Justicia simplex*, MTT assay

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Arachnid bites and microbial infections can cause dermonecrotic lesions and other skin diseases. Excessive and continuous use of antibiotics for treatment leads to the emergence of antibiotic-resistant strains, as well as various side effects. Additionally, prescribed skin ointment medications are prohibitively expensive for the general public. Social stigma often prevents timely medical care, complicating conditions and making them more difficult to treat later. Furthermore, cancer remains a severe health problem and is the second-leading cause of death worldwide. Despite the development of numerous anticancer drugs, they do not fully and satisfactorily address the treatment or control of cancer. Natural products from plants based on our traditional knowledge could provide a more affordable and accessible option for the general population.

Traditional herbal medicine is rapidly gaining popularity in developed countries. In China, it constitutes between 30% and 50% of all medications, and in countries such as Nigeria, Ghana, Zambia, and Mali, it is the first choice for 60% of children suffering from high malarial fever[1]. Since the isolation of codeine and narcotine from *Papaver somniferum*, numerous phytocompounds have been extracted from plants, many of which are used in pharmaceuticals. For instance, dehydroabietic acid from *Pinus elliottii*, ellagic acid from *Rosa rugosa*, and triterpenes from *Planchonia careya*, as well as various other plant extracts, have been identified as antimicrobial agents against multidrug-resistant bacteria[2]. The use of plants as anticancer agents has been documented in many traditional medicinal systems, and numerous phytocompounds have been scientifically validated for their anticancer properties[3]. Clinically used plant-derived anticancer drugs such as taxol, vinblastine, and topotecan exhibit various molecular mechanisms of action[4]. Additionally, the synergistic effects and multiple modes of action of compounds in plant extracts offer a promising approach to combating drug

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resistance. For example, whole plant therapy using *Artemisia annua* has been reported in animal models as an alternative to isolated artemisinin, aiming to overcome artemisinin resistance[5,6].

Justicia is the largest genus within the Acanthaceae family, and many of its species are noted for their medicinal properties, including anti-cancer effects. For example, fusicoserpenol A and dolabeserpenoid acid A are antifungal compounds isolated from the leaves of *Hypoestes serpens*, another member of the Acanthaceae family[7]. The leaves of various Acanthaceae plants are also known for their use in external wound healing. Additionally, several species of *Justicia* have been employed in treating inflammation-related conditions such as eczema, rheumatism, lumbar pain, and swelling[8]. Notably, whole plant extracts of *J. spicigera* have been reported to exhibit cytotoxic effects on leukemia cells[9].

Many traditional medicinal plants remain unexplored or undisclosed by healers who aim to protect their knowledge and prevent commercialization. *Justicia simplex* D. Don is known to have numerous ethnopharmacological uses but lacks scientific research. Our report highlights that *J. simplex* D. Don has been traditionally employed by folk medicine practitioners, known as "Vaidyans," in Thiruvananthapuram district, Kerala, India, for treating dermonecrotic arachnidism caused by spider bites and other skin lesions. The present descendant of the practitioners revealed the recipe for the drug preparation. Plant specimens were collected early in the morning, kept in humid conditions, and ground in water, likely to preserve volatile compounds. The preparation involved mixing the ground plant slurry with milk. Patients are instructed to take this medicine for three days or longer, while adhering to a vegetarian diet and avoiding salty and spicy foods.

Scientific evaluation and the identification of novel, potent compounds in plant extracts are crucial steps in exploring herbal medicines for new drug development. Techniques such as FTIR, HRMS, GCMS, and NMR can be employed to identify these novel or potent compounds. This study aims to assess the wound healing, antibacterial, antifungal, and anticancer properties of the ethyl acetate extract of *J. simplex* leaves. Additionally, it seeks to identify the bioactive compounds present in the extract and predict their pharmacological activities and drug-likeness.

Materials and Methods

Plant extract preparation

The leaves of *J. simplex* were collected from nearby areas within the Thiruvananthapuram district of Kerala. (altitude 18 m, latitude 8°29′07″ N, and longitude 76°56′57″ E). The leaves were dried in the shade and finely ground using a flour mill grinder. The powdered material (100 g) was then extracted with ethyl acetate using a Soxhlet apparatus, filtered, and concentrated to dryness (1.3 g). Plant species identification was carried out by Dr. V. Viji from the Department of Botany at Government College for Women, Thiruvananthapuram. A voucher specimen (no. 215) has been deposited in the college herbarium. The plant name was checked at http://www.worldfloraonline.org/taxon/wfo-0001033277 (Accessed on 01/05/2023).

In vitro scratch assay

The *in vitro* scratch wound assay was performed according to the method of Liang *et al.*[10] with minor modifications, using a RAW cell line (monocyte/macrophage-like cells) obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in DMEM supplemented with additives. Test samples were prepared in cell culture-grade DMSO and diluted with DMEM to concentrations of 15, 20, and 25 µg/mL. A Millipore syringe filter with a pore size of 0.2 µm was then used to filter the mixture. Both control and treated wells were incubated at 37°C and photographed at various intervals.

In vitro evaluation of antimicrobial activities

Antibacterial and antifungal activities were studied by the agar well diffusion method. Gram positive *Staphylococcus aureus* (ATCC 25923), *Streptococcus mutans* (ATCC 25175) and gram negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) bacterial strains were used. The fungal strains used were *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231). The diameter of clear zone of inhibition was measured against test culture.

DPPH radical scavenging assay

The radical scavenging activity of ethyl acetate extract of leaves of *J. simplex* was evaluated by using DPPH according to the method reported by Chang *et al.*[11] with slight modifications. Different volumes (1.25, 2.5, 5, 10 and 20 µL) of plant extracts were

made up to a final volume of 30 µL with DMSO and 1.47mL DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. Then the absorbency of the mixture was read at 517 nm using an ELISA reader. DPPH solution was taken as control. The inhibition ratio (in percent) was calculated as follows

$$\% \text{ of inhibition} = \frac{\text{absorbance of control - absorbance of test}}{\text{Absorbance of control}} \times 100$$

Anticancer evaluation

Cell culture

HT-29 cell line is procured from NCCS, Pune, India. HT- 29 is a human colorectal adenocarcinoma cell line with epithelial morphology. This colon cell lines were maintained in DMEM.

Cell seeding

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 µL cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Anticancer assay by direct microscopic observation

Different concentrations of the leaf extract (6.25, 12.5, 25, 50 and 100 µg/mL) were prepared in 5% DMEM using a cyclomixer. The sample solutions were then filtered through 0.22 µm Millipore syringe filter to ensure sterility.

After 24 h of growth, the medium was removed, 100 µL of freshly prepared plant extract was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator along with control. After 24 h, the cells were observed under an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and photographed.

Anticancer assay by MTT method

Anticancer assay using MTT was done following the method by Talarico *et al*.[12].

Determination of apoptosis by acridine orange and ethidium bromide double staining

DNA binding dyes acridine orange and ethidium bromide were used for the morphological detection of apoptotic and necrotic cells[13]. The stained cells were washed twice with 1X PBS and observed under a fluorescence microscope using blue filter (Olympus CKX41 with Optika Pro5 camera).

Identification of bioactive compounds

Identification of bioactive compounds present in the extract was done by FTIR and HRMS analyses followed by literature review.

Drug- likeness of the compounds

Physicochemical characters and drug-likeness of the compounds were predicted using SWISSADME[14].

Prediction of pharmacological activities

Chemdraw MDL Molfile (MOL) formats of the compounds were used to predict the pharmacological activities employing PASS online software (http://way2drug.com/passonline/predict.php)[15].

Statistical analysis

The statistical significance of data for control and treated groups was assessed by analysis of variance using SPSS 13 for Windows. Statistical significance was accepted when $p \leq 0.05$.

Results

Wound healing activity

The wound healing efficiency was found to be both concentration and time dependent. At a concentration of 20 µg/mL, wound closure was completed within 36 h, while at 25 µg/mL, cell migration was achieved within 24 h of treatment (Fig. 1).

Antimicrobial activities

The ethyl acetate extract of leaves of *J. simplex* exhibited significant growth inhibitory activity against the four strains of pathogenic bacteria and the two

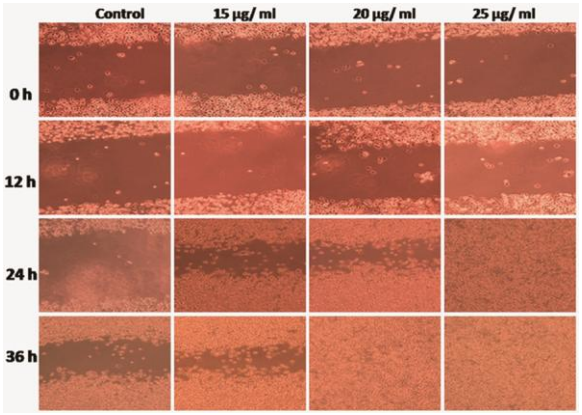


Fig. 1 — Scratch wound healing assay showing the effect of ethyl acetate extract of leaves of *J. simplex* on RAW cells at various concentrations

strains of pathogenic fungi tested based on their concentration gradient, MICs ranged from 12.5 to 25.0 mg/mL (Table 1, Fig. 2). The MIC value of the fungi and bacteria except *S. mutans* is 12.5 mg/mL. For *S. mutans*, it is 25 mg/mL.

DPPH radical scavenging assay

A significant increase in the scavenging activity (IC_{50} value of 13.53 µL) was observed with an increase in the concentration of the plant extract (Fig. 3).

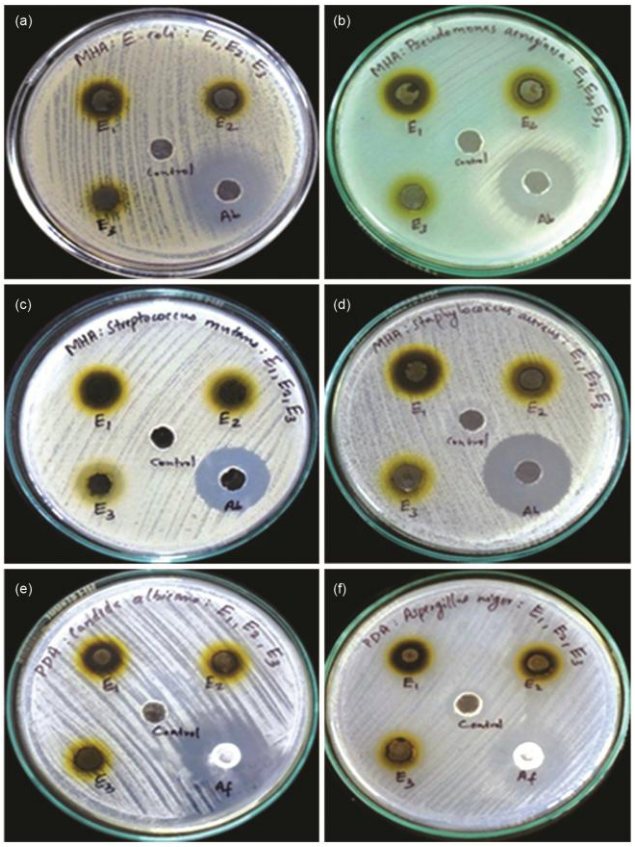


Fig. 2 — Antimicrobial activity of ethyl acetate extract of leaves of *J. simplex*. (a) *E. coli*, (b) *P. aeruginosa*, (c) *S. mutans*, (d) *S. aureus*, (e) *C. albicans*, (f) *A. niger*
Concentrations: E_1 = 25 mg/mL, E_2 = 12.5 mg/mL, E_3 = 6.25 mg/mL

Cell viability assays

Viability of HT-29 cells was assayed by measuring the activity of cellular enzymes that reduce MTT to formazan. (Fig. 4) shows the cytotoxic effect of ethyl acetate extract of *J. simplex* at various concentrations. The value of negative control is taken as 100% and viability of the treated groups were calculated relative to the control. Viability of the cells decreases with increasing concentration of the extract from 80.23±1.66% at 6.25 µg/mL to 46.18±1.54 at 100 µg/mL.

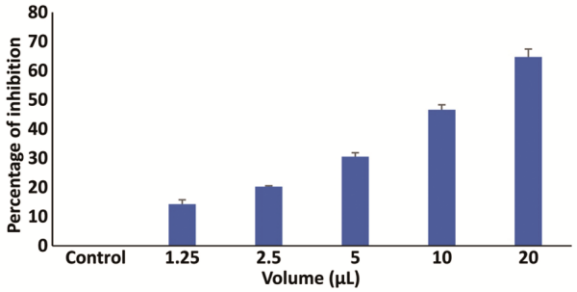


Fig. 3 — Free radical scavenging activity of ethyl acetate extract of leaves of *J. simplex* measured by DPPH assay. Each value is a mean of three separate determinations ± SE, all values significant at 0.01 level when compared with control

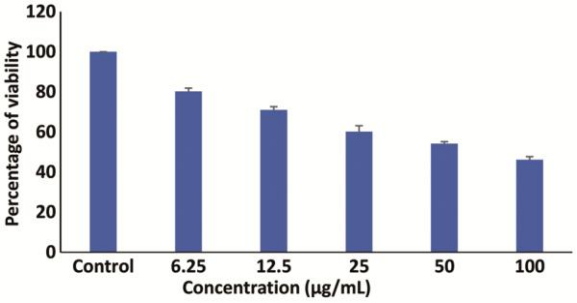


Fig. 4 — Viability of HT-29 cells measured by MTT assay. Each value is a mean of three separate determinations ± SE, all values are significant at 0.01 level when compared with control

Table 1 — Antibacterial and antifungal activities of ethyl acetate extracts of *J. simplex* leaves

Microbial strains		Inhibition zone (mm) Concentration			
		Control	6.25 mg/mL	12.5 mg/mL	25 mg/mL
Gram +ve	*Streptococcus mutans*	-	-	-	14.33±0.66*
bacteria	*Staphylococcus aureus*	-	-	9.33±0.88*	14.33±0.67*
Gram –ve	*Escherichia coli*	-	-	13.67±0.88*	14.67±0.88*
bacteria	*Pseudomonas aeruginosa*	-	-	15.00±0.57*	19.00±1.15*
Fungi	*Aspergillus niger*	-	-	14.00±0.57*	15.00±1.00*
	Candida albicans	-	-	11.67±0.67*	13.33±0.88*

*Significance at 0.01 level when compared with control
- - no activity, values are mean ± SE of three replicates

The phase contrast microscopy images showed cytotoxic effects of the extract, such as distortion of cell shape, rupture of cell membrane, release of cytoplasmic granules and vacuolation (Fig. 5).

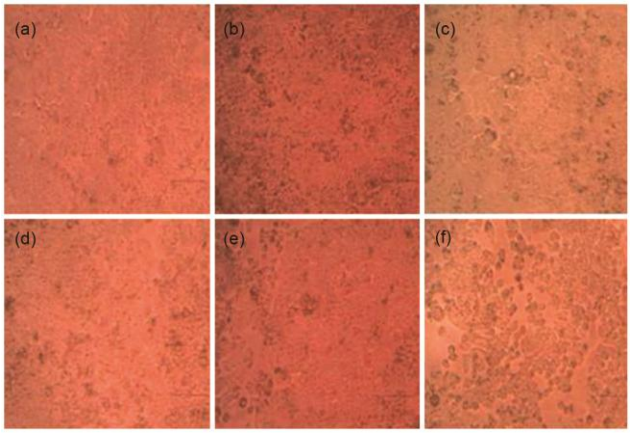


Fig. 5 — Cytotoxic changes in HT-29 cells treated with ethyl acetate extract of leaves of *J. simplex*. The concentration of the extracts used for treatment is as follows. (a) control, (b) 6.25 µg/mL, (c) 12.5 µg/mL, (d) 25 µg/mL, (e) 50 µg/mL, (f) 100 µg/mL

Cell apoptosis

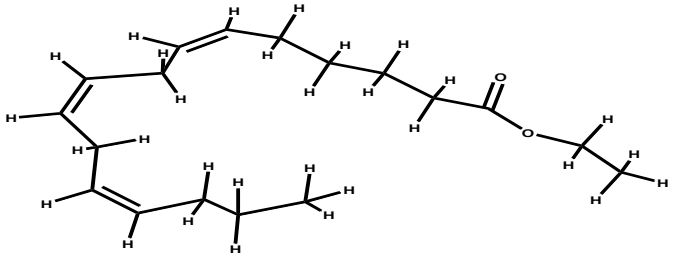
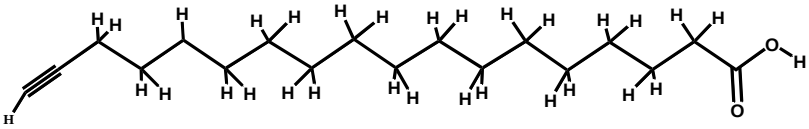
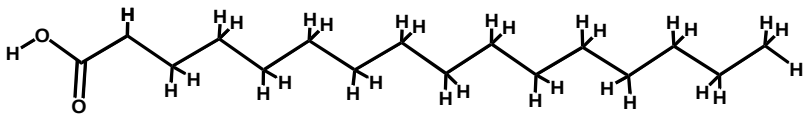
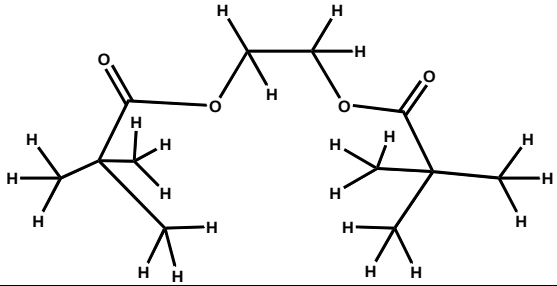
Acridine orange and ethidium bromide double staining showed apoptotic effects of ethyl acetate extract of leaves of *J. simplex* (Fig. 6). It was observed that in the control, normal cells with green fluorescence were evenly distributed due to the presence of acridine orange. An increase in red fluorescence was observed in culture with the extract of *J. simplex* due to ethidium bromide staining.

Identification of bioactive molecules

FTIR and HRMS data (Supplementary Fig. S1 & Fig. S2) and a comparative literature review[16] confirmed the presence of four major compounds in the ethyl acetate extract of *J. simplex* (Table 2).

FTIR peaks from 1610 to 1680 cm^{-1} indicate the presence of C=C stretching of alkene, peak at 1730 cm^{-1} indicates C=O stretching of α,β-unsaturated ester bond, peak at 1210 cm^{-1} indicates C-O stretching. The presence of C=C bonds, C=O and C-O stretching and HRMS spectrum peak at m/z 277.23 its comparison with previous literature indicate the compound as ethyl 6,9,12-hexadecatrienoate ($C_{18}H_{30}O_2$, exact mass

Table 2 — Compounds identified in the ethyl acetate extract of leaves of *J. simplex*

Sl. No:	Compounds	Molecular formula	Molecular mass	Structure of the compound
1	Ethyl 6,9,12-hexadecatrienoate	$C_{18}H_{30}O_2$	278.22	
2	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280.24	
3	n- Hexadecanoic acid	$C_{16}H_{32}O_2$	256.24	
4	Ethylene glycol 0,0-di(pivaloyl)	$C_{12}H_{22}O_4$	230.15	

278.22). Weak peaks from 2100 to 2260 cm^{-1} indicate C≡C stretching, peak for O-H in the range of 2500 cm^{-1} to 3300 cm^{-1} and a C=O stretch in the range of 1730 cm^{-1} indicate the presence of a carboxylic group (-COOH). The presence of these functional groups and mass comparison with the HRMS peak at 279.23 indicate the presence of 17-octadecynoic acid ($C_{18}H_{32}O_2$, exact mass 280.24). The -COOH peaks in FTIR and the m/z HRMS peak at 256.23 indicate the presence of n-hexadecanoic acid ($C_{16}H_{32}O_2$, exact mass 256.24). C-O stretching and C=O peaks in the FTIR and HRMS peak at 229.14 m/z indicate the presence of ethylene glycol 0,0-di(pivaloyl) ($C_{12}H_{22}O_4$, exact mass 230.15).

Physicochemical characters and drug likeness of the identified compounds

SWISSADME analysis gave the molecular weight of ethyl 6, 9, 12-hexadecatrienoate as 278.43 g/mol, having twenty heavy atoms, thirteen rotatable bonds, and two hydrogen bond acceptors, molar refractivity 88.50 and TPSA 26.30 Å. It is a derivative of the unsaturated fatty acid decenoic acid, formed by esterification. The molecular weight of 17-octadecynoic acid as 280.45 g/mol, twenty heavy atoms, fifteen rotatable bonds, two hydrogen bond acceptors and one hydrogen bond donor, molar refractivity 88.57 and TPSA 37.30 Å. It is a derivative of the saturated, straight chain fatty acid, decanoic acid. The molecular weight of n-hexadecanoic acid acid as 256.42 g/mol, eighteen heavy atoms, fourteen rotatable bonds, two hydrogen bond acceptors and one hydrogen bond donor, molar refractivity 80.80 and TPSA 37.30 Å. It is also an decanoic acid derivative. The molecular weight of ethylene glycol 0,0-di(pivaloyl) as 230.30 g/mol, sixteen heavy atoms, seven rotatable bonds and four hydrogen bond acceptors, molar refractivity 61.85 and TPSA 52.60 Å. It is an esterified compound of glycol and pivalic acid. Except for ethyl 6, 9, 12-hexadecatrienoate, all other compounds obey the Lipinski rule of drug likeness.

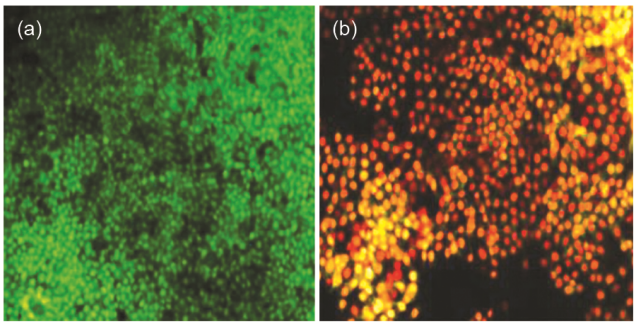


Fig. 6 — HT-29 cells stained by acridine orange and ethidium bromide, (a) normal cells showing green fluorescence, (b) cells treated with ethyl acetate extract of *J. simplex* show red florescence (conc. 50 µg/mL, magnification:400x)

Prediction of pharmacological activities

All the compounds were predicted to have anti-inflammatory anti-eczematic and anti-neoplastic activities as their probability to be active (Pa) is higher than probability to be inactive (Pi) (Table 3). Pa>0.7 indicates that all the compounds are very likely to exhibit anti-eczematic activity in the experiment and high chances of analogous to the known pharmaceutical agent[17]. Regarding anti-inflammatory activity, ethyl 6, 9, 12-hexadecatrienoate and ethylene glycol 0,0-di(pivaloyl) have very high activity (Pa>0.7), whereas 17-octadecynoic acid and n-hexadecanoic acid are likely to exhibit the activity in experiment and are unlike known pharmaceutical agents (0.5<Pa<0.7).

Discussion

The results of the current study demonstrate the effectiveness of *J. simplex* against pathogenic microbes and wound healing activity, evaluating the traditional usage of the plant against skin diseases. The scratch-wound assay is used to measure cell migration *in vitro* after a thin wound is introduced into a confluent monolayer of cells. Cells at the margin of the wound polarise and move inside the wound area. It mimics the migration of cells *in vivo* and enables to study cell to cell interactions[10]. The

Table 3 — Pass analysis of the phytocompounds present in the ethyl acetate extract of *J. simplex*

Compounds	Anti-inflammatory		Anti-eczematic		Anti-neoplastic	
	Pa	Pi	Pa	Pi	Pa	Pi
Ethyl 6,9,12-hexadecatrienoate	0.764	0.009	0.963	0.002	0.265	0.034
17-Octadecynoic acid	0.522	0.051	0.872	0.007	0.200	0.085
n- Hexadecanoic acid	0.515	0.052	0.920	0.004	0.226	0.059
Ethylene glycol 0,0-di(pivaloyl)	0.705	0.015	0.804	0.018	0.389	0.005

Pa- pharmacological activity, Pi- Pharmacological inactivity

result of the study indicates the presence of phytocompounds in the ethyl acetate extract of *J. simplex* that can induce cell proliferation and migration. The extract is also proven to have antioxidant activity. The presence of antioxidant compounds in the extract augments the wound healing process. The extracts of *Marantodes pumilum* have antioxidant effects and are shown to have faster wound healing effects in rats[18]. Curcumin, N-acetyl cysteine, quercetin and chitosan are antioxidant compounds that have been proved to enhance wound healing activity in *in vitro* and animal model studies[19].

Bacteria and fungi are primary contributors to skin infections and can develop resistance to antibiotics and antifungal treatments, respectively. In this study, the ethyl acetate extract of *J. simplex* demonstrated antimicrobial activity against these pathogens. The pathogens examined are known to cause superinfections in COVID-19 patients[20]. Primary and secondary cutaneous aspergillosis occur in people with weakened immune systems[21]. Candida species is one of the most prevalent species causing skin infections[22]. These observations substantiate the traditional use of *J. simplex* as a therapeutic agent for skin diseases.

Reactive oxygen species (ROS) produced within the cells as a result of various metabolic activities can damage proteins, lipids and DNA. The antioxidants find these free radicals, neutralize them, protect cells and make them less susceptible to becoming cancerous. An increase in the ROS-antioxidant activity ratio causes oxidative stress, which induces abnormal cell division and cancer. Plants have developed various antioxidant mechanisms that protect the cells from the harmful effects of oxidative stress[23]. In the current study, DPPH assay proves the antioxidant activity of ethyl acetate extract of *J. simplex*. Furthermore, MTT assay proves the anticancer efficacy of the extract against HT-29 human colorectal adenocarcinoma cells. The apoptotic effect of the extract is indicated by DNA damage and increase in red fluorescence in malignant cells subsequent to treatment with DNA binding dyes. Acridine orange is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into DNA. Ethidium bromide is taken up only by non-viable cells with damaged membranes and emits red fluorescence. These results signify the conventional use of *J. simplex* for therapeutic purposes. The anticancer activities of *J. simplex* in cellular and animal models have been reported[24]. The cytotoxic activity against human lung epithelial cells and other pharmacological effects of *J. procumbens*, which has been used in traditional Chinese medicine for cancer, have been reported[25,26]. The anti-inflammatory and antinociceptive activities of *J. gendarussa* in mice, as well as the pharmacological evaluation of *J. neesii*, were studied[27,28]. The antitussive effect of *J. adhatoda*, in combination with other plant extracts in specific proportions, was clinically studied in patients with acute upper respiratory tract infections[29].

Identifying phytocompounds in bioactive extracts is a crucial step in herbal therapy. With the exception of ethylene glycol 0,0-di(pivaloyl), which is a dihydric alcohol, all other compounds identified in the extract from this study are ester derivatives of the fatty acids decanoic acid and decenoic acid. Antibacterial activity of ethylene glycol, antibacterial and antifungal efficacies of water soluble decanoic acid formulations were proved[30,31]. Topical application of the fatty acid palmitoleic acid has been found to hasten the wound healing process[32]. Fatty acids are also important in diminishing various inflammatory skin diseases[33]. These studies demonstrate that the ethyl acetate extract of *J. simplex* comprises biologically active compounds that produce synergistic effects, supporting its use in folk medicine. The plant has been traditionally used as an oral remedy for an extended period, suggesting its safety and lack of toxicity. For drug-likeness, the Topological Polar Surface Area (TPSA) should fall between 20 and 130 Å[14]. The TPSA of the compounds in the study extract ranges from 26 to 53 Å, reflecting the bioactivity of these individual compounds.

Conclusion

The current study highlights the synergistic effects of various compounds in *J. simplex*, supporting its use as a therapeutic agent in folk medicine and suggesting potential long-lasting benefits as a natural product. The plant shows promise as a source for novel drug discovery; however, further research is needed, including the isolation of active molecules, their bioassays, chemical modifications to enhance their efficacy, and their synthesis.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_24(4)(2025)310-318_SupplData.pdf

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

S.S: the investigation, formal analysis and the original draft preparation. G.J: methodology, resources, software. P.K: conceptualization, editing and supervision.

Ethics Approval

Not applicable

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and also in the supplementary material. The data may also be provided by the corresponding author upon reasonable request.

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