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ORIGINAL ARTICLE

Phytochemical characterization and evaluation of antioxidant, antimicrobial, antibiofilm and anticancer activities of ethyl acetate seed extract of *Hydnocarpus laurifolia* (Dennst) Sleummer

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Abstract
Various functional groups were observed in the FTIR analysis of *Hydnocarpus laurifolia* seeds ethyl acetate extract such as O–H, N–H, C–H, $–CH_2$, O=C=O, C=O, C=O–NH, and CH_3, etc. Eleven bioactive compounds were detected via GC–MS and the predominant compounds include (1S)-2-cyclopentene-1-tridecanoicacid (chaulmoogric acid) (80.59%); 2-cyclopentene-1-undecanoic acid (hydnocarpic acid) (6.76%); cyclobutylamine (5.28%); methyl thioacetate (ethanethioic acid) (4.84%); lignoceric acid (2.21%). The TPC and TFC values were 0.110 ± 0.04 GAE g^{-1} and 0.175 ± 0.05 g CE g^{-1} respectively. Ethyl acetate extract showed strong DDPH free radical scavenging activity with IC_{50} value 10.64 ± 0.48 µg ml^{-1} and antioxidant activity index 3.759. The ethyl acetate extract also exhibited potential ABTS radical scavenging efficacy with a very low IC_{50} value, i.e., 07.81 ± 0.48 µg ml^{-1}. *P. aeruginosa* was the most sensitive bacteria to the extract with 33.16 ± 0.88 mm inhibition zone and MIC: 3.12 mg ml^{-1}, MBC: 6.25 mg ml^{-1}. *P. aeruginosa* biofilm was inhibited by ethyl acetate extract 99.22% at MIC concentration. The LM images displayed a decrease in the number of biofilm cells and FE-SEM micrographs showcased the extensive decrease as well as disintegration in biofilm. Additionally, ethyl acetate extract was found selectively cytotoxic to the K562 cancer cells having an IC_{50} of 25.41 µg ml^{-1} and barely cytotoxic to normal PBMCs having an IC_{50} of 482.54 µg ml^{-1}, and the selectivity index value was 18.99. Data validate scientifically the traditional use of *H. laurifolia* seeds in folk medicines and confirmed that it can be used in modern phytomedicines as an antioxidant, antimicrobial, antibiofilm, and anticancer agent and is toxicologically safe.

Keywords *Hydnocarpus laurifolia* · FTIR · GC–MS · Antioxidant activity · Antimicrobial activity · Antibiofilm activity · Anticancer activity

Introduction

United Nations Conference on Trade and Development stated that around 30% of the drugs manufactured and prescribed globally are produced by medicinal plants and utilized in treating several disorders as spasms, pain, diabetes, inflammation, cancer, neurological diseases, parasitic as well as microbial infections, etc. (Anand et al. 2019). Microbial infections are a significant burden to the economic stability of societies and public health around the world. More than 400 million deaths per year worldwide are recorded due to microbial pathologies, which is a higher burden than cardiovascular or cancer disorders (Fitzpatrick et al. 2019).

Since the last century, despite the considerable improvement in global health care, millions of people have no access to quality health services or are impoverished because of high health care expenses (Rajput and Akash 2020). Medicinal plants derived herbal products have contributed significantly to the discovery of novel phyto-compounds with significant pharmacological activities, but it is strongly required to validate their toxicological safety and therapeutic efficacies to provide characteristic information for drug development and to ensure safe doses for the prescription (De

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Veras et al. 2020). Based on this premise, various plants, herbs, and shrubs used in conventional medicare systems have been investigated for their pharmacological and therapeutic aspects so that they can be utilized to treat various conditions as microbial infections, inflammation, cancer, etc. (Branquinho et al. 2017; Rajput and Kumar 2020a).

Furthermore, biofilm is one of the most virulent pathogenic factors of microbial strains causing various infections and facilitating their survival even in hostile conditions. The antibiotic resistance of biofilms has emerged as a serious problem for researchers across the world (Mishra et al. 2020). The antibiofilm agents are the class of bioactive compounds that can prevent the formation of biofilm or aggregation of cells, thereby aiding in antimicrobial penetration (Landini et al. 2010). Synthetic antibiofilm agents, combinational therapies, and antagonistic hybrids could not achieve the desired treatment results due to resistance, ineffectiveness, and side effects. Biofilm development is a complicated procedure that involves various steps that can be targeted by natural antibiofilm agents. They either act synergistically or solely by several mechanisms of action to prevent biofilm formation (Mishra et al. 2020).

Besides, the free radicals produced during our normal metabolism process and their excessive amounts cause cellular as well as tissue damage via oxidative stress (Tonisi et al. 2020). Antioxidant compounds act as free radical scavengers and slow down or inhibit lipid peroxidation or other free radical-mediated processes. Therefore, they tend to protect from various diseases via inhibiting oxidative damage (Rashid et al. 2013). In recent years, medicinal plant-derived natural antioxidant agents, and their mechanism of action have attracted the interest of researchers as various studies indicating towards side effects related to the consumption of synthetic antioxidant compounds (Lourenço et al., 2019).

A major dilemma in treating patients with cancer is the severe side effects of chemotherapeutic cytotoxic drugs (Fong et al. 2016). Nowadays, the development of cytotoxic drugs by natural compounds has been extensively explored as medicinal plants-derived bioactive molecules are easy to isolate, available in abundance, safe due to low cytotoxicity, and have fewer side effects as compared to synthetic chemopreventive drugs for the treatment of cancer and anticancer drug discovery (Kumar et al. 2019).

Hydnocarpus laurifolia (Dennst) Sleummer. (Synonym: *Hydnocarpus pentandra* (Buch-Ham) Oken. or *Hydnocarpus wightiana* Blume) belongs to the family Flacourtiaceae. It is widely distributed in Indian tropical forests of the Western Ghats and from Maharashtra coast to Kerala, Tripura, Assam and is abundantly grows in Sri Lanka also (Jadav et al. 2016). It has been traditionally utilized by communities in south India as a remedy for treating helminthiasis, intestinal worms, infected wounds, leprosy, fever, leucoderma, piles, skin diseases, dermatitis, eczema, bronchopathy, verminosis, tubercular laryngitis, ulcers, wounds with inflammation, and decoction or paste is useful in infectious vaginal secretion (Sini et al. 2005a; Reddy et al. 2013). However, most of such therapeutic properties of *H. laurifolia* have not been scientifically evaluated yet.

Recently, researchers have made several efforts in discovering novel antimicrobial, antioxidant, and anticancer phyto-compounds from several biological sources. Several medicinal plants are having significant biological activities and from them, new drugs have been derived and approved. Due to the resistance of microbial strains an increasing emergence of antibiotic-resistant microorganisms has been recorded, therefore, it is essential to cease the spread of multiple drug-resistant (MDR) microorganisms and to improve the treatment methods. In recent years there are several cases are reported of failure and side effects of chemotherapy in cancer patients. Thus, there is an urgent need of searching not only for improved versions of existing drugs but also for novel therapeutic compounds that have a new mechanism of action or targets (Arulmozhi et al. 2018).

In this sense, this investigation aims to ensure and validate the scientific basis of the use of *H. laurifolia* seeds in the traditional medicinal system. In this context, the present research aims to evaluate the phytochemical composition, antioxidant, antimicrobial, antibiofilm, and cytotoxicity of ethyl acetate seed extract of *H. laurifolia*.

Material and methods

Plant material and extraction

The certified seed material of *H. laurifolia* was purchased from Herbal Health Research Consortium Ltd., Punjab, India in March 2016. For the removal of dust particles and moisture content, seeds were rinsed with tap water and dried in shade at room temperature, respectively, then seed powder was prepared with the help of an electric grinder. In the Soxhlet extractor, 200 g powder was used for the extraction by immersing in 600 ml of ethyl acetate. The crude seed extract was evaporated by Khera Vacuum Rotary Evaporator, KI-102 to get solvent-free seed extract and stored at 4 °C in the sterile vial for further use (Rajput and Kumar 2020b).

FTIR spectral analysis

Fourier transform infrared (FTIR) spectroscopy of ethyl acetate seed extract of *H. laurifolia* was conducted to identify the functional groups using Bruker Tensor 37 instrument. The infrared spectrum of the extract was analyzed between the wavelength of 4000–600 cm^{-1} and important absorption peaks appeared in fingerprinting as well as in functional group regions were studied for the assignment of functional

groups, chemical bonding, and vibrational bands present in the extracts.

Phytochemical component evaluation by GC–MS

GC–MS evaluation of the *H. laurofolia* seeds ethyl acetate extract was carried out using Gas Chromatography-Mass Spectrometer (Perkin Elmer GC Clarus 500) with an Elite-5MS capillary column. Further analytical conditions and precautions were followed as described by Rajput et al. (2021). The phyto-components were identified based on the analysis of mass spectra and retention time of particular peak to the already reported computer database of the NIST and Wiley libraries or with the help of literature (Rajput et al. 2021).

Total phenolic content (TPC) and total flavonoid content (TFC) estimation

The Folin-Ciocalteu colorimetric assay was used to estimate TPC according to Chaovanalikit and Wrolstad (2004) and The TFC was estimated according to Dewanto et al. (2002). Gallic acid (GAE) and Catechin (CE) were taken as reference phenolic and flavonoid compounds, respectively.

DPPH free radical scavenging activity

DPPH (2,2-diphenyl1-picrylhydrazyl) free radical scavenging potential of the extract was checked by the methodology described by Scherer and Godoy (2009) with some minor modifications. 3 ml of freshly prepared DPPH working solution (0.004% w/v) was suspended into 2 ml of various concentrations of the extract (7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000 µg ml^{-1}) followed by incubation in dark at room temperature for 30 min. 3 ml DPPH solution + 2 ml ethanol was taken as negative control and Ascorbic acid was utilized as a reference standard to compare the absorbance of the extract at respective concentrations and OD was recorded at 517 nm. The IC_{50} value was obtained via the graphical method through best-fit line plotted concentration versus % inhibition. The experiment was performed in triplicates and the % inhibition was calculated by the following equation:

$$\%\text{Inhibition} = \frac{(\text{OD of control} - \text{OD of sample})}{(\text{OD of control})} \times 100$$

The antioxidant activity index (AAI) was measured by the following formula:

$$\text{AAI} = \frac{\text{Final concentration of DPPH radical}}{\text{IC50}}$$

AAI values for extract were recognized according to the: poor antioxidant efficacy < 0.05 < moderate < 1.0 < strong < 2.0 < very strong (Scherer and Godoy 2009).

ABTS radical scavenging activity

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic-acid) radical scavenging efficacy was performed according to González-Palma et al (2016) with some minor modifications. 7 mM concentration of ABTS in water was prepared for the stock solution and ABTS radical cations were prepared by dissolving 2.45 mM Potassium persulfate in stock solution. The final reaction mixture was incubated at room temperature in dark for 12–16 h, further diluted with the help of distilled water to an approximate 0.70 ± 0.02 absorbance, at 734 nm. 0.07-ml concentration of the extract was suspended in 3 ml of final ABTS radical reaction mixture followed by incubation for 6 min and OD was recorded at 734 nm. The IC_{50} value of the extract was measured with the help of a graphical representation plotted against concentration versus % inhibition. The antioxidant potential was estimated using the following equation and compared with ascorbic acid:

$$\%\text{Inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

Microbial strains and culture media

The antimicrobial efficacy of *H. laurofolia* seeds ethyl acetate extract was assessed against three bacteria *Pseudomonas aeruginosa* (MTCC 2474), *Staphylococcus epidermidis* (MTCC 435), *Staphylococcus aureus* (MTCC 1144), two yeast *Candida albicans* (MTCC 227), *Malassezia furfur* (MTCC 1374), and dermatophyte *Microsporum audouinii* (MTCC 8197) procured from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. The active microbial cultures for the experimental use were prepared in Mueller–Hinton Broth (MHB) (bacteria), Sabouraud Dextrose Broth (SDB) (*C. albicans, M. audouinii*), and Emmons modified Sabouraud Dextrose Broth (mSDB) (*M. furfur*) by transferring inoculum from stocks. Microorganisms were incubated in a BOD incubator in the following conditions: bacteria at 37 °C for 24 h, *M. audouinii* at 25 °C for 7 days, *C. albicans* at 25 °C for 48 h, and *M. furfur* at 30 °C for 7 days.

Antimicrobial susceptibility test

Agar well diffusion assay is used to perform antimicrobial activity (Ahmad et al. 1998). The final concentration (200 mg/ml) of the extract was prepared by dissolving in Dimethyl sulfoxide (DMSO) and it was taken as the

negative control. To compare the antibacterial efficacy of the extract, bacitracin (8 units/disk), neomycin (10 µg/disk), and erythromycin (15 µg/disk) were taken, similarly, to compare antifungal potential, terbinafine (1 µg/disk), ketoconazole (10 µg/disk), and griseofulvin (10 µg/disk) were used by disk diffusion assay (Hudzicki 2009). Poisoned Food Technique was used to estimate the antifungal potency of the extract against *M. audouinii* (Nene and Thapliyal 1993) and the inhibition percentage of the fungal mycelium was calculated by the equation:

$$\%\text{Mycelial inhibition} = \frac{\text{Mycelial growth in control} - \text{Mycelial growth in treatment)}}{\text{Mycelial growth in control}} \times 100$$

Minimum inhibitory concentration (MIC), minimum bactericidal and fungicidal concentration (MBC/MFC)

MIC of the extract was estimated by performing the broth microdilution susceptibility assay and different concentrations were prepared (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 mg ml^{-1}). Ketoconazole and Bacitracin (5–0.039 mg ml^{-1}) were taken as the positive controls, whereas broth and DMSO were utilized as the negative control. The lowest concentration that inhibits microbial population is considered MIC. For the MBC/MFC determination, 10-µl aliquots from the MIC titer plate were transferred to the freshly prepared agar plates and incubated at 37 °C for 24 h. MBC/MFC was estimated by observing the minimum concentration of extract that allows no visible growth on the surface of the agar plate (Wiegand et al. 2008).

Time-kill assay

The killing efficacy of the seed extract was analyzed by the time-kill assay, according to Sheh-Hong and Darah (2013). 1 ml of the stock culture (1.5×10^8 CFU ml^{-1}) with various concentrations of the ethyl acetate seed extract (½×, 1×, and 2×MIC) were supplemented in 50 ml of MH broth followed by incubation at 37 °C, with constant shaking (150 rpm/min) for 48 h. Broth + inoculum + DMSO was considered a negative control and broth + extract (inoculum free medium) was used to measure the turbidity of the extracts at each concentration. About 3 ml of the portion from the reaction mixture was withdrawn at every 4 h intervals and the optical density (OD) at 540 nm was taken to monitor the killing efficacy of the extract. The assay was performed in triplicates and to evaluate the killing potency over time, growth profile curves were plotted.

Antibiofilm activity by ring test

The qualitative antibiofilm efficacy of the extract was performed by the ring test assay. 5 ml of MH broth was mixed with 100 µl of bacterial inoculum followed by incubation for 24 h at 37 °C statically, then varying extract concentrations (1/8×, ¼×½×, 1×MIC) were supplemented into the test tubes and incubated for further 24 h. After incubation, tubes were twice rinsed with distilled water, and mature biofilm attached as a ring on the walls of the test tubes was stained with crystal violet (Nithyanand et al. 2015).

Biofilm inhibitory concentration (BIC) determination

The quantitative antibiofilm efficacy estimation of the extract was carried out by the 96-well microtiter plate assay (Kalishwaralal et al. 2010). 180 µl of MH broth was filled in the wells and 10 µl of bacterial culture (1.5×10^8 CFU ml^{-1}) was inoculated into it followed by incubation for 24 h at 37 °C for the preparation of biofilm. After that, 10 µl from the various concentrations of the extract (0.048, 0.097, 0.195, 0.39, 0.78, 1.56, and 3.12 mg ml^{-1}) were supplemented to the wells following incubation for 24 h. Then planktonic bacterial cells were discarded gently and loosely adherent cells were rinsed twice and air-dried. 0.1% (w/v) Crystal violet was utilized to stain the biofilm attached to the well surface and ethanol (95%) was used to solubilize the stain. Broth + inoculum + DMSO (extract free medium) was taken as control. OD of the samples was taken at 620 nm with the help of an ELISA reader. Percentage biofilm inhibition was measured by the following equation:

$$\%\,\text{Biofilm Inhibition} = \frac{\text{Absorbance of non treated control} - \text{absorbance of extract treated sample}}{\text{absorbance of non treated control}} \times 100$$

Antibiofilm activity evaluation by microscopy

Light microscopy (LM) and field emission scanning electron microscopy (FE-SEM) have been carried out to visualize the changes in the morphology of the biofilm upon the treatment of extract. 1 ml broth and 1% inoculum were suspended into the wells of 24-well plates, then 1×1 cm glass slides were carefully placed into the wells and incubated at 37 °C for 24 h for the formation of biofilm. Then varying concentrations of the extract (¼×½×, 1×MIC) were immersed into the wells followed by incubation for 24 h. Further, glass slides were

washed with phosphate-buffered saline (PBS). For LM, slides were stained with crystal violet solution (0.1%) for 1 min then examine at 100X magnification with the help of a light microscope (RH-93UXL Unilab, India). The glass slides were fixed for FE-SEM with the help of 2% glutaraldehyde at 4 °C for 8 h; washed with PBS; dehydrated with ethanol (20, 40, 60, 80, 100%), then coated with gold, and analyzed in Carl Zeiss Ultra Plus FE-SEM (Subramenium et al. 2015).

Anticancer activity on K562 cells

The anticancer activity of the extract was performed on K562 cancer cells (ATCC CCL-243) obtained from National Centre For Cell Science (NCCS), Pune, India. The inhibitory effect was analyzed via MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Malaikozhundan et al. 2017). RPMI-1640, the cell growth medium with fetal bovine serum (10%) maintained at 37 °C in a humid atmosphere of CO_2 (5%). 50 µl of cell suspension was seeded in titer plate wells following overnight incubation at 37 °C. Subsequently, 50 µl of various concentrations of extract (200, 100, 50, 25, and 12.5 µg ml^{-1}) were suspended in the wells and incubated for 24 h at 37 °C. After incubation, the MTT reagent (100 µl) was mixed with the reaction mixture following incubation of 3 h. After gently discarding of culture medium, formazan crystals were solubilized in DMSO and the absorbance was recorded at 570 nm. The drug Thymol was taken as positive control and non-treated cell culture was taken as the negative control. The dose–response graph was plotted and the IC_{50} value was obtained. The cell viability percentage and inhibition percentage were calculated by the following equations:

$$\%\text{Cell viability} = \frac{\text{OD of treated cells} - \text{OD of blank}}{\text{OD of control cells} - \text{OD of blank}} \times 100$$

$$\%\text{Cell Inhibition} = 100 - \%\text{cell viability}$$

Cytotoxicity on normal human peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from the fresh blood samples and cells were separated by a Ficoll density gradient centrifuge (400×*g*) at 20 °C for 40 min. The undisturbed lymphocytes were carefully harvested, rinsed twice with PBS, pelleted, then resuspended in the cell growth medium having 10% FBS (v/v) (Dutra et al. 2012). The cytotoxicity of varying extract concentrations was measured as mentioned for the K562 cells via MTT assay.

Selectivity index (SI)

The SI is the ratio of the cytotoxicity on the normal cells to the cytotoxicity on the cancer cells and if SI was recorded as < 1, it is considered toxic or non-selective, between the range of 1–10 (weakly selective), and > 10 is classified as non-toxic or safe to use (Ogbole et al. 2017).

Statistical analysis

All the in vitro activities were carried out in three biological replicates and expressed as mean ± standard error. One-way ANOVA was applied to analyze data using SPSS (Statistics Version 20) to evaluate the significant mean differences and the data was considered significant at $p < 0.05$.

Results

FTIR spectral analysis

The IR spectra of *H. laurifolia* seed ethyl acetate extract were analyzed for the identification of important functional groups responsible for its biological activities. Peaks were observed in the functional group as well as fingerprinting region, indicated towards the specific functional groups present in the extract (Fig. 1). The functional group and skeletal vibrations assignment in the ethyl acetate extract were: 3738.07 (O–H and N–H stretching), 3004.87 (C–H stretching), 2921.51 (stretching of $–CH_2$ of acyl chains), 2854.08 ($–CH_2$ stretching of the methylene chains), 2358.48 (O=C=O stretching), 1742.78 (C=O stretching), 1644.30 (C=C stretching), 1456.53 (CH_3 bending), 1367.59 (C–O stretching, C–H deformation, N–H deformation), 1156.77 (C–O stretching), and 719.36 (C–H bending). These functional groups indicated the possible presence of several bioactive metabolites in the extracts as phenolics, amines, alkanes, aldehydes, and esters.

Analysis of phytochemical composition by GC–MC

Eleven phytoconstituents (Table 1) were identified by the GC–MS of *H. laurifolia* seeds ethyl acetate extract and the GC-chromatogram is presented in Fig. 2. (1S)-2-Cyclopentene-1-tridecanoicacid (Chaulmoogric acid) was the most abundant phytochemical compound reaching as high as 80.59% followed by 2-cyclopentene-1-undecanoic acid (hydnocarpic acid) (6.76%); cyclobutylamine (5.28%); methyl thioacetate (ethanethioic acid) (4.84%); lignoceric acid (2.21%); β-sitosterol glucoside (sitogluside) (1.31%); *Z*,*Z*-3,11-octadecadien-1-ol acetate (1.08%); 1-Heptanol (0.56%); 9,12-octadecadien-1-ol, (Z,Z) (0.54%); hexadecanoic acid (palmitic acid) (0.50%); and luteolin (0.20%).

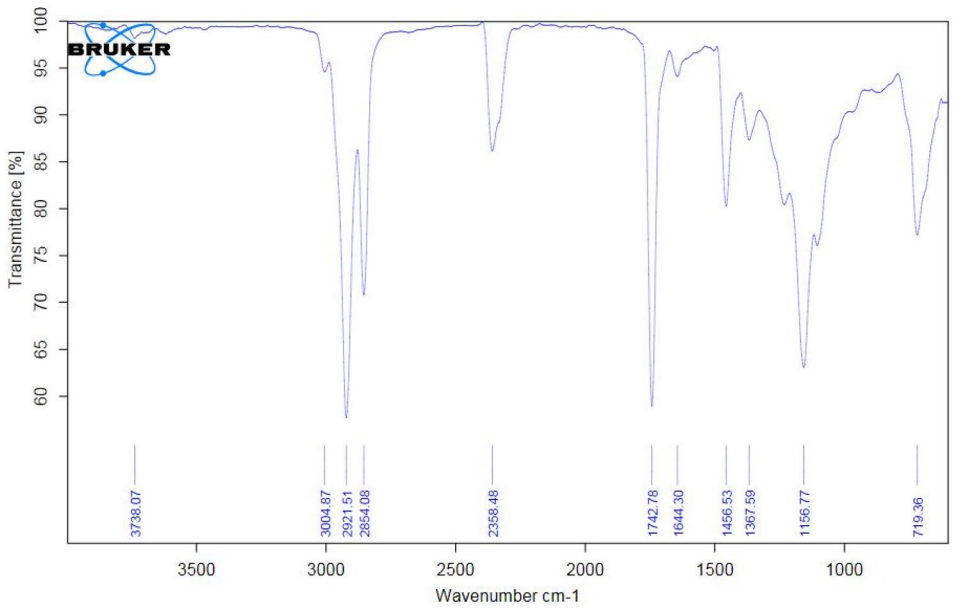


Fig. 1 FTIR spectrum analysis of *H. laurifolia* seeds ethyl acetate extract

TPC, TFC, DPPH, and ABTS radical scavenging activity

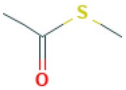
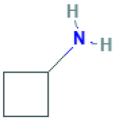
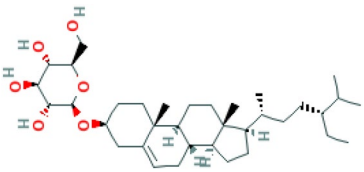
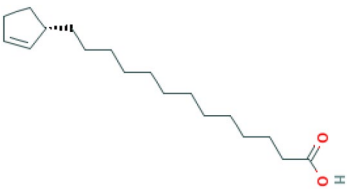
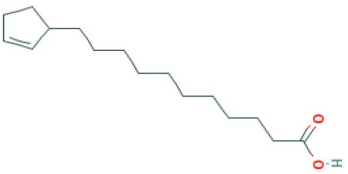
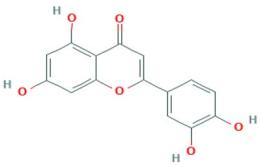
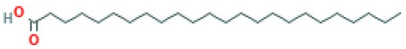
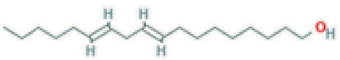
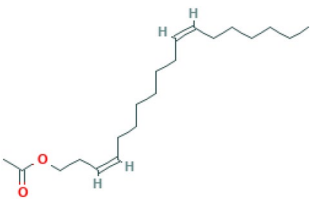
The linear regression equation of gallic acid for the TPC determination was $y = 0.0057x + 0.0224$, $R^2 = 0.998$. The TPC of seed ethyl acetate extract was 0.110 ± 0.04 g GAE g^{-1} ($p < 0.05$). The linear regression of catechin for the TFC evaluation was $y = 0.003x + 0.053$; $R^2 = 0.998$. The TFC of ethyl acetate seed extract was 0.175 ± 0.05 g CE g^{-1} ($p < 0.05$). The % DPPH radical scavenging efficacy of ethyl acetate extract is shown in Fig. 3. At 1000 µg ml^{-1} concentration of ascorbic acid, 97.57 ± 0.47% inhibition was observed, and at the same concentration of extract, 92.94 ± 0.97% inhibition was reported that was comparable to the ascorbic acid, so considered as a potential antioxidant, with a very low IC_{50} value, i.e., 10.64 ± 0.48 µg ml^{-1} and antioxidant activity index (AAI), i.e., 3.759. The percent ABTS radical scavenging activity of *H. laurifolia* seeds ethyl acetate extract is shown in Fig. 4. The extract showed potential ABTS radical scavenging efficacy with a very low IC_{50} value, i.e., 07.81 ± 0.48 µg ml^{-1}. The *H. laurifolia* seeds ethyl acetate extract possess high TPC and TFC content that probably correspond to a very strong antioxidant activity.

Antimicrobial efficacy, MIC, and MBC/MFC

Table 2 showed the results of antimicrobial activity of *H. laurifolia* ethyl acetate seed extract and reference antimicrobials. *P. aeruginosa* among all the test strains found most sensitive to ethyl acetate seed extract with an inhibition zone of 33.50 ± 0.33 mm followed by *S. epidermidis* (17.50 ± 0.28 mm), *S. aureus* (16.50 ± 0.33 mm), *M. furfur* (15.50 ± 0.38 mm), and *C. albicans* (13.00 ± 0.33 mm). The reference antibiotic neomycin exerted the best efficacy against *P. aeruginosa* with a 21.05 ± 0.38 mm inhibition zone; erythromycin showed the best activity against *S. aureus* with a 19.33 ± 0.38 mm zone of inhibition, bacitracin showed the most potential efficacy against *S. epidermidis* with a 19.00 ± 0.38 mm inhibition zone. The sensitivity of *C. albicans* and *M. furfur* was also compared to the standard antifungal drugs, and it was reported that both the fungal pathogens were resistant to the griseofulvin. Ketoconazole was effective against both *C. albicans* and *M. furfur* with 19.50 ± 0.38 mm and 15.50 ± 0.33 mm inhibition zone, respectively, whereas, terbinafine was only effective against *M. furfur* with an 11.05 ± 0.33 mm inhibition zone. Ethyl acetate extract of *H. laurifolia* seeds inhibited 88.11% of mycelial growth of *M. audouinii.* The reference antifungal terbinafine showed the best antifungal activity against the dermatophyte *M. audouinii* with a percentage inhibition of 86.50% followed by griseofulvin (59.40%) and ketoconazole (38.31%).

The MIC of the ethyl acetate extract ranges between 3.12–50 mg ml^{-1} and the range of MBC/MFC lies between 6.25–100 mg ml^{-1} against all test pathogens. The MIC of ethyl acetate extract was observed lowest against *P. aeruginosa* and *M. audouinii,* i.e., 3.12 mg ml^{-1} followed by *S. epidermidis* (6.25 mg ml^{-1}), *S. aureus* (12.5 mg ml^{-1}), *M. furfur* (12.5 mg ml^{-1}), and *C. albicans* (50 mg ml^{-1}). The MBC/MFC of ethyl acetate extract was found lowest against *P. aeruginosa* and *M. audouinii,* i.e., 6.25 mg ml^{-1} followed by *S. epidermidis* (12.5 mg ml^{-1}), *S. aureus* (25 mg ml^{-1}), *M. furfur* (50 mg ml^{-1}), and *C. albicans* (100 mg ml^{-1}).

Table 1 The phytochemical characterization of *H. laurifolia* ethyl acetate seed extract

Retention time (Min.)	Compound	Molecular weight (g/mol)	Molecular formula	Peak area (%)	Structure of the compound
3.10	1-Heptanol	116.2	$C_7H_{16}O$	0.56	
4.05	Methyl thioacetate (ethanethioic acid)	90.15	C_3H_6OS	04.84	
4.78	Cyclobutylamine	71.12	C_4H_9N	05.28	
29.54	β-sitosterol glucoside (sitogluside)	576.8	$C_{35}H_{60}O_6$	01.31	
33.96	(1S)-2-Cyclopentene-1-tridecanoi-cacid (chaulmoogric acid)	280.4	$C_{18}H_{32}O_2$	80.59	
34.72	2-Cyclopentene-1-undecanoic acid (hydnocarpic acid)	252.39	$C_{16}H_{28}O_2$	06.76	
35.04	Hexadecanoic acid (palmitic acid)	256.42	$C_{16}H_{32}O_2$	0.50	
36.96	Luteolin	286.24	$C_{15}H_{10}O_6$	0.20	
39.10	Lignoceric acid	368.6	$C_{24}H_{48}O_2$	02.21	
41.60	9,12-Octadecadien-1-ol, (*Z*,*Z*)	266.5	$C_{18}H_{34}O$	00.54	
43.07	*Z*,*Z*-3,11-Octadecadien-1-ol acetate	308.5	$C_{20}H_{36}O_2$	01.08	

Time-kill assay

As *P. aeruginosa* was the most sensitive strain to *H. laurifolia* seed extract, the time-killing test was only performed against it. *P. aeruginosa* was exposed to various extract concentrations (½ × MIC, 1 × MIC, and 2 × MIC) and non-treated control (Fig. 5a, b, c, d). A rise in OD of bacterial growth was observed in the initial

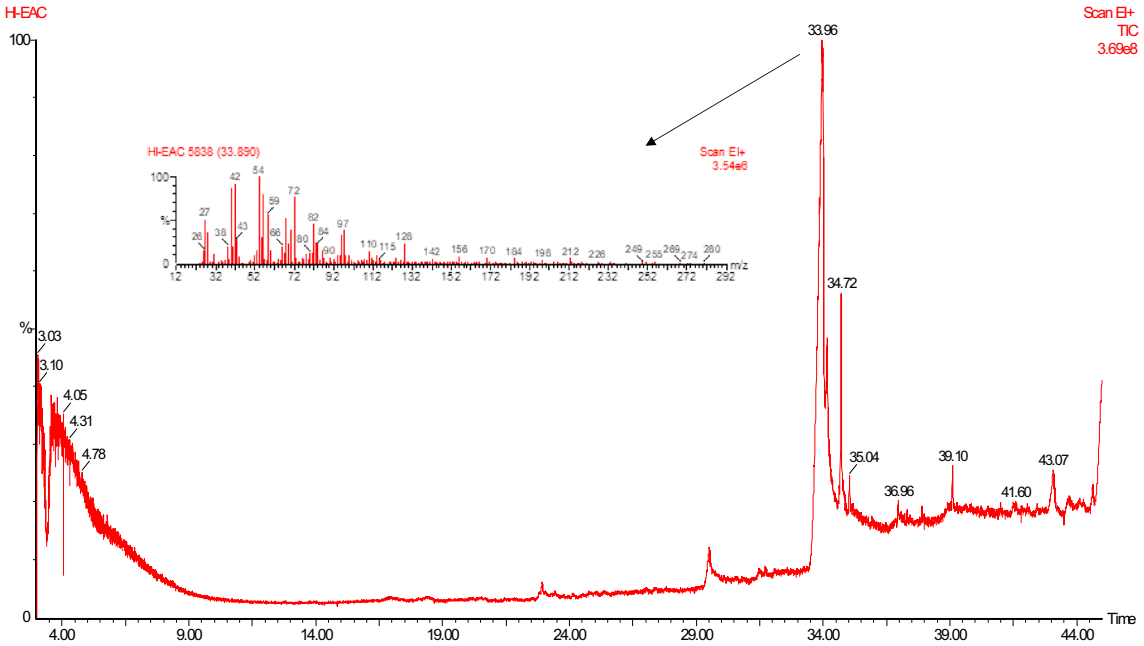


Fig. 2 GC–MS chromatogram of *H. laurifolia* seeds ethyl acetate extract

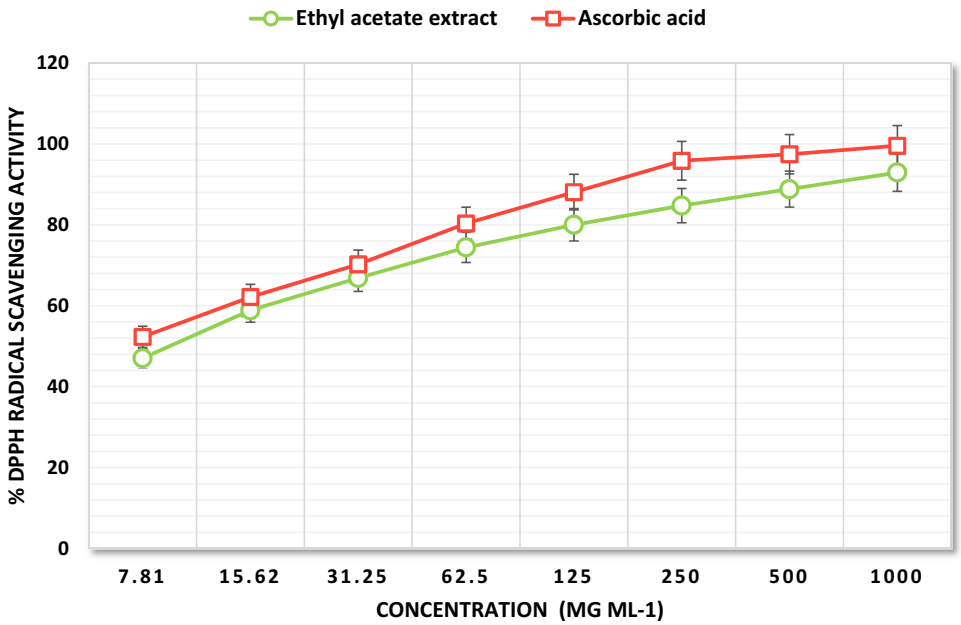


Fig. 3 DPPH radical scavenging activity of *H. laurifolia* seeds ethyl acetate extract

20 h then a slight drop in OD on the exposure of ½ × MIC (1.56 mg ml^{-1}) (Fig. 5 a) was reported when compared to the non-treated control (Fig. 5d) and led to the stationary phase. However, at the exposure of MIC (3.12 mg ml^{-1}) concentration (Fig. 5b), initially, a small rise in OD was observed for 4 h then a large drop in OD in comparison to control was observed which showed bacterial eradication just after 4-h exposure of the extract. At the treatment of 2 × MIC (6.25 mg ml^{-1}) (Fig. 5c) of extract, there was a large drop in OD reported in comparison to non-treated control, which indicated bacterial death just after the exposure to 2 × MIC. Time-kill curves explained the efficacy of ethyl acetate seed extract of *H. laurifolia* to inhibit *P. aeruginosa* cells (bacteriostatic) at the ½ × MIC and eradicating cells (bactericidal) at the MIC or higher concentrations.

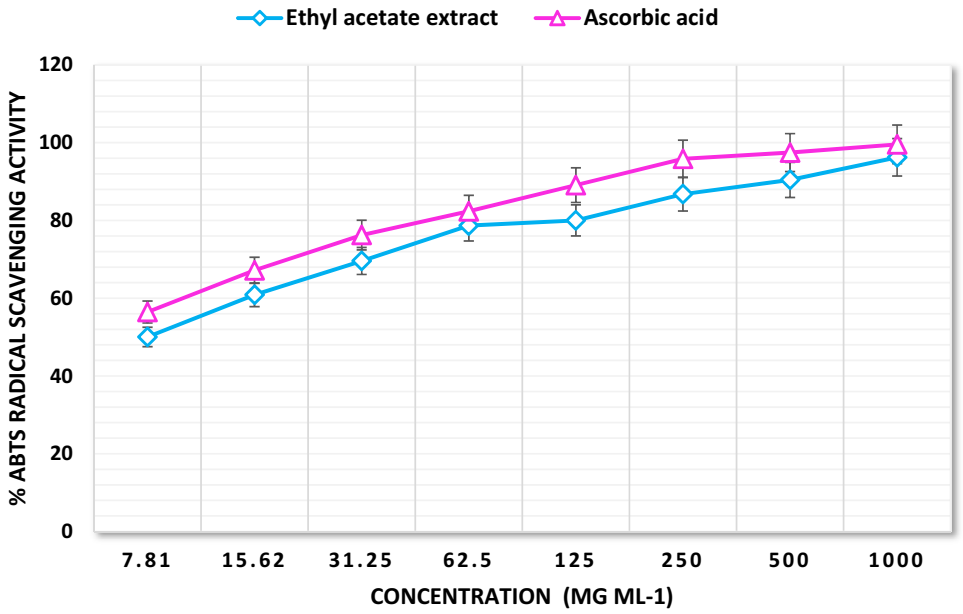


Fig. 4 ABTS radical scavenging activity of *H. laurifolia* seeds ethyl acetate extract

Table 2 Diameter of inhibition zones of *H. laurifolia* ethyl acetate seed extract and reference antimicrobial agents against some MTCC pathogens

Extract/reference	Diameter of Inhibition Zone (mm)				
	Bacteria			Fungi	
	Pseudomonas aeruginosa (MTCC 2474)	*Staphylococcus epidermidis* (MTCC 435)	*Staphylococcus aureus* (MTCC 1144)	*Malassezia furfur* (MTCC 1374)	*Candida albicans* (MTCC 227)
Ethyl acetate	33.50 ± 0.33	17.50 ± 0.28	16.50 ± 0.33	15.50 ± 0.38	13.00 ± 0.33
Neomycin	21.50 ± 0.38	17.50 ± 0.28	17.00 ± 0.33	NT	NT
Bacitracin	16.50 ± 0.33	19.00 ± 0.38	12.00 ± 0.33	NT	NT
Erythromycin	18.50 ± 0.38	14.00 ± 0.33	19.33 ± 0.38	NT	NT
Griseofulvin	NT	NT	NT	–	–
Terbinafine	NT	NT	NT	11.05 ± 0.33	-
Ketoconazole	NT	NT	NT	15.50 ± 0.33	19.50 ± 0.38
DMSO	–	–	–	–	–

Values are the mean of three biological replicates; diameter of well: 6 mm; significant ($P \leq 0.05$)

DMSO dimethyl sulfoxide, *MTCC* microbial type culture collection, *NT* not tested, - no zone of inhibition

Antibiofilm activity by ring test

There was a dose-dependent reduction in biofilm formation around the walls of the test tubes was observed at 1/8×, ¼×½×, and 1×MIC concentrations of ethyl acetate seed extract of *H. laurifolia* when compared to non-treated control (Fig. 6). It was reported that, upon the treatment of MIC, there was no biofilm or biofilm ring present on the wall of the test tube. It indicated that ethyl acetate extract of *H. laurifolia* possessed significant antibiofilm potential against *P. aeruginosa* biofilm.

Biofilm inhibitory concentration

On the exposure to various concentrations of *H. laurifolia* ethyl acetate extract, a dose-dependent decrease in biofilm formation was detected that confirmed the significant efficacy of ethyl acetate extract on *P. aeruginosa* biofilm. 3.12 mg ml^{-1} concentration of extract exerted 99.22% *P. aeruginosa* biofilm inhibition, so it was taken as minimum biofilm inhibitory concentration (MBIC) (Fig. 7).

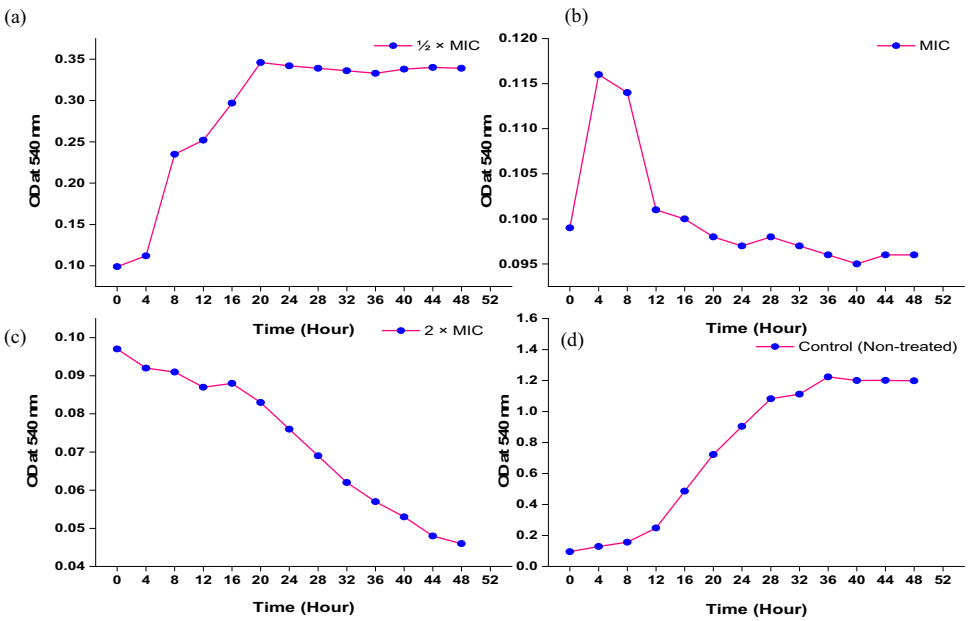


Fig. 5 Growth curves of *P. aeruginosa* on the exposure of *H. laurifolia* seeds ethyl acetate extract. **a** 1.56 mg ml^{-1}, **b** 3.12 mg ml^{-1}, **c** 6.25 mg ml^{-1}, and **d** non-treated control

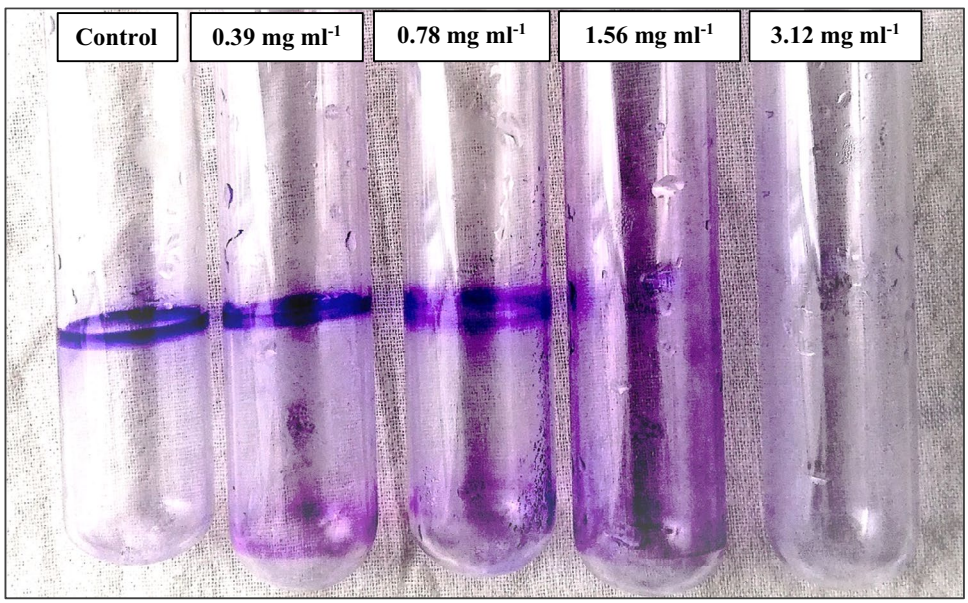


Fig. 6 Antibiofilm activity of *H. laurifolia* seeds ethyl acetate extract against *P. aeruginosa* biofilm by ring assay

Microscopy of antibiofilm efficacy

The light microscopic investigation displayed the dose-dependent increase in antibiofilm activity of ethyl acetate extract (¼ × ½ ×, 1 × MIC concentrations) on *P. aeruginosa* biofilm. LM images clearly showed that on the exposure of various concentrations of extract, there was a well-defined reduction in *P. aeruginosa* biofilm cells as compared to non-treated control (Fig. 8a, b, c, d).

To examine the morphological alterations induced in *P. aeruginosa* biofilm on the exposure of different concentrations (¼ × ½ ×, 1 × MIC) of ethyl acetate extract, the slides were visualized under FE-SEM. Figure 9a (non-treated control) showed normal to smooth cell surface morphology of *P. aeruginosa* biofilm, whereas, ethyl acetate extract-treated biofilm showed less cell number, cell modulation as extensive blebbing, pronounced membrane wrinkling as well as cell debris in comparison to non-treated control (Fig. 9b, c, d). Most of the biofilm cells have lost their membrane integrity and their cellular matrix was arising out from the cells, possibly because of cellular content leakage from the degraded bacterial cell membrane. Such observations indicated the membrane-dependent mechanism of action

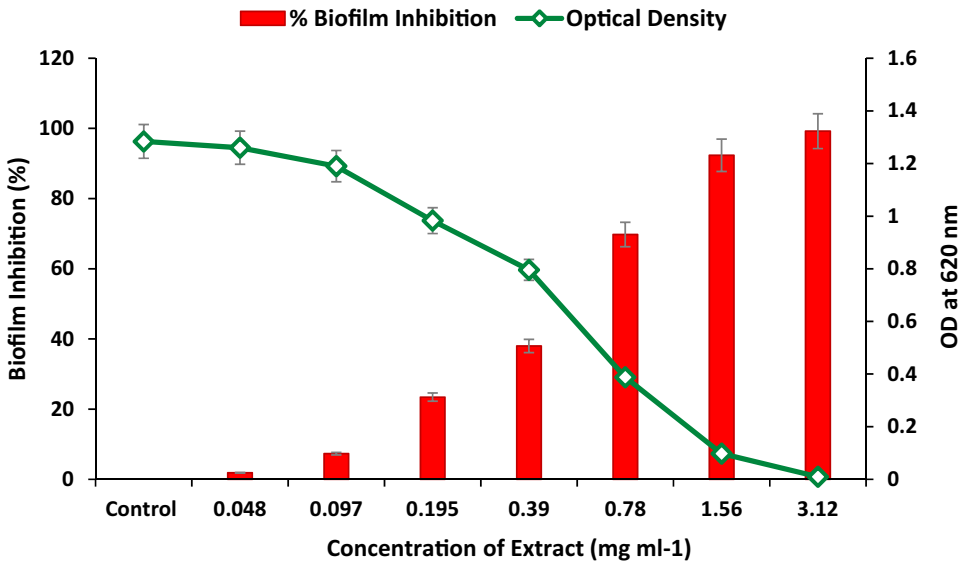


Fig. 7 Percentage inhibition of *P. aeruginosa* biofilm by *H. laurifolia* seeds ethyl acetate extract

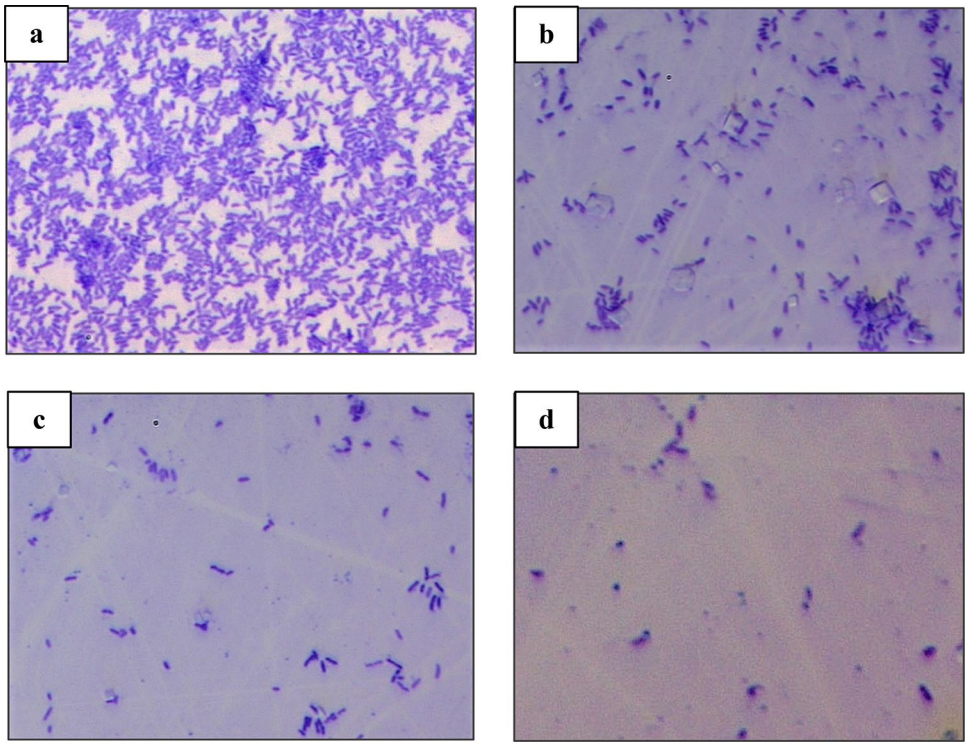


Fig. 8 Light microscopy (100 X) of *P. aeruginosa* biofilm treated with **a** non-treated control, **b** 0.78 mg ml^{-1}, **c** 1.56 mg ml^{-1}, **d** 3.12 mg ml^{-1} concentration of *H. laurifolia* seeds ethyl acetate extract

of antibiofilm efficacy of *H. laurifolia* seeds ethyl acetate extract against *P. aeruginosa*.

Cytotoxicity evaluation on K562 and PBMC cells

The anticancer activity of *H. laurifolia* ethyl acetate extract was tested on the K562 cancer cells and the dose-dependent efficacy is displayed in Fig. 10. The results of the MTT bioassay showed the decreased cell viability as well as inhibition in cell proliferation in a dose-dependent manner as plant extract showed the concentration-dependent cytotoxic effect on the K562 cell line. It was observed that an increase in extract concentration respectively increase the cell inhibition percentage and at the lowest concentration (12.5 µg ml^{-1}), cell proliferation or viability was highest (70.36%). The highest cell inhibition percentage (82.07%) was reported at 200 µg ml^{-1} of extract concentration with the IC_{50} of 25.41 µg ml^{-1}.). The IC_{50} of ethyl acetate extract was lower than that of thymol (IC_{50} value 0.089 µg ml^{-1}) but, it displayed significant inhibition in K562 cells proliferation on

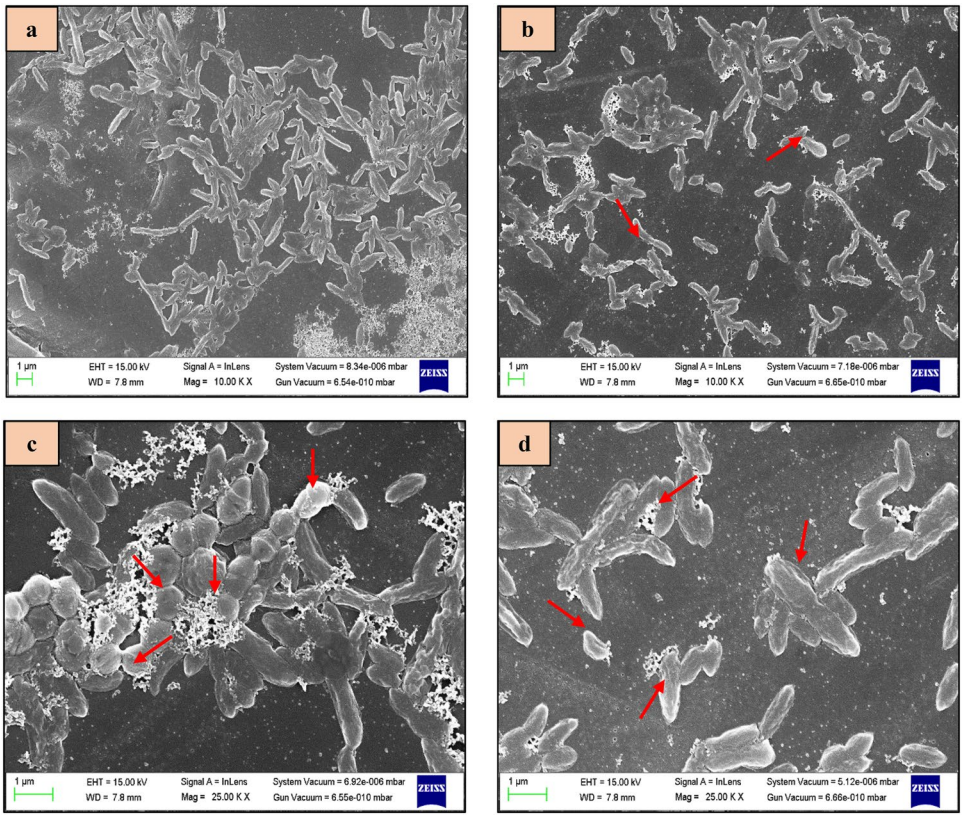


Fig. 9 FE-SEM micrographs of *P. aeruginosa* biofilm treated with, **a** non-treated control, **b** 0.78 mg ml^{-1}, **c** 1.56 mg ml^{-1}, **d** 3.12 mg ml^{-1} concentration of *H. laurifolia* seeds ethyl acetate extract

the exposure to increasing concentrations of the extract. This indicates clearly that the treatment of ethyl acetate extract on K562 cancer cells significantly altered the metabolic processes of cells that contribute to the cell viability and enlighting its potential anticancer activity.

Whereas, the treatment of *H. laurifolia* ethyl acetate seed extract on normal PBMC cells did not display strong cytotoxicity and the percentage of cell viability and cell inhibition were shown in (Fig. 11). The IC_{50} value was 482.54 µg ml^{-1} as compared to the IC_{50} of thymol, i.e., 113.86 µg ml^{-1}. The SI value was 18.99 that presented *H. laurifolia* seed ethyl acetate extract was safe to use for further analysis.

Discussion

Medicinal plants, a widespread source of therapeutically important extracts have biologically active phytochemicals that provide important leads for the development of novel, environment-friendly herbal medicines having negligible side effects compared to chemical drugs and lead to standardized phytomedicines. In modern medicine, plants utilized in traditional and folk medicines have been accepted as leads for therapeutic drug development (Rajput and Kumar 2020a; Singh and Navneet 2021; Gupta et al. 2019). Hence, the present piece of work was concentrated on the phytochemical analysis of ethyl acetate seed extract of *H. laurifolia* and its several biological activities (antimicrobial, antibiofilm, antioxidant, anticancer). Ethyl acetate is used for the extraction process because of its biological as well as chemical characteristics, as low toxicity and medium polarity that enable efficient extraction of both non-polar and polar phytochemicals (Marino 2005).

FTIR technique is used for the functional group identification of any sample depending on peaks observed in the infra-red spectrum. It is a unique method that shows the chemical fingerprint of any sample, and there will be no two compounds having identical spectra of FTIR (Easmin et al. 2017). The functional groups identified in the extracts indicated the presence of several probable phytocompounds like alkaloids, phenols, lipids, flavonoids, fatty acids, proteins, amino acids, carbohydrates, etc. However, only FTIR analysis of the extract is not sufficient enough to identify the various classes of metabolites (Bhat et al. 2018). The present investigation reported the presence of several biologically active functional groups as N–H, C–H, O–H, O=C=O, $–CH_2$, C=O, C=O–NH, and CH_3, etc. that indicated the presence of alkanes, phenolics, ester, amines, and aldehydes in the extracts.

The present investigation reported the presence of (1S)-2-cyclopentene-1-tridecanoicacid (chaulmoogric acid); 2-cyclopentene-1-undecanoic acid (hydnocarpic acid); cyclobutylamine; methyl thioacetate (ethanethioic acid);

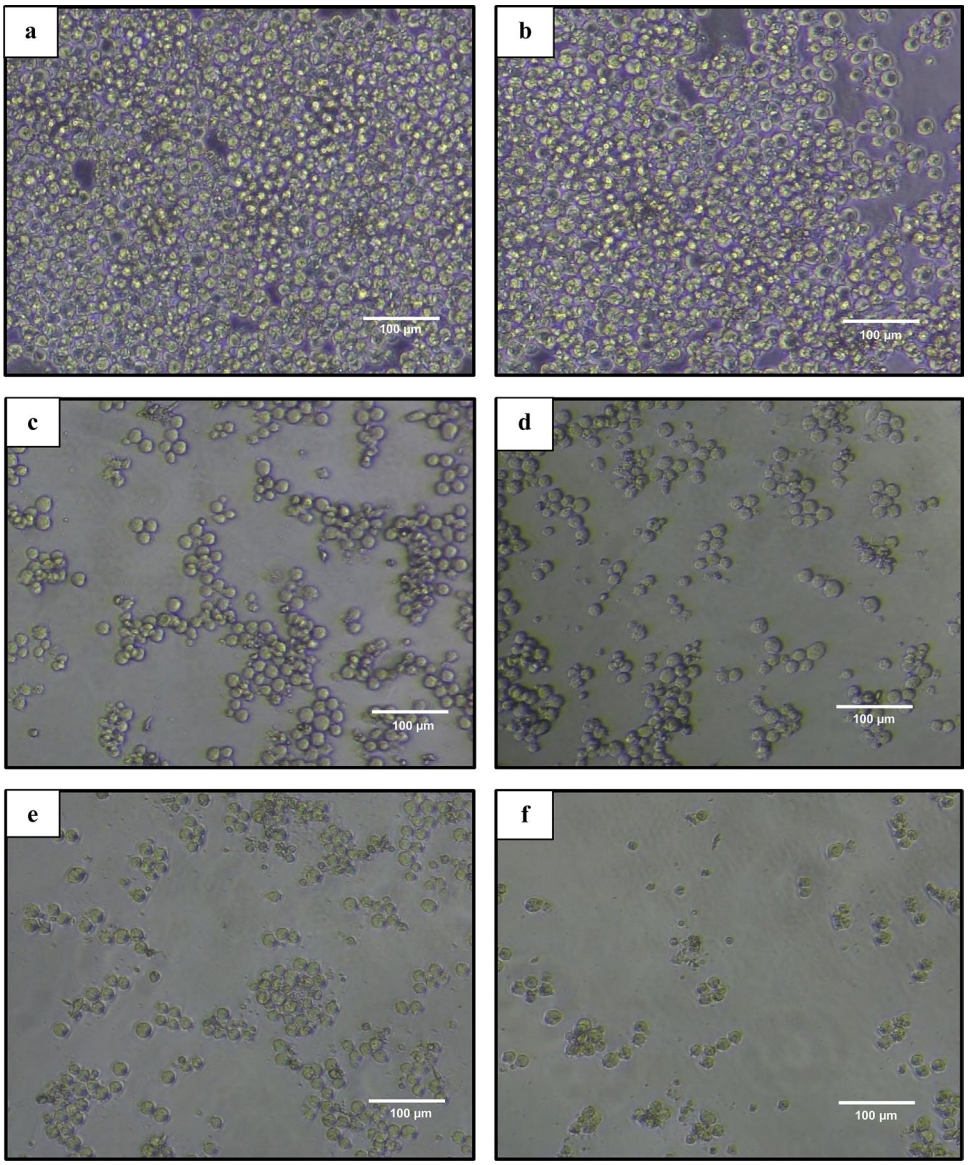


Fig. 10 Cytotoxicity of different concentrations, **a** Non-treated control, **b** 12.5 µg ml^{-1}, **c** 25 µg ml^{-1}, **d** 50 µg ml^{-1}, **e** 100 µg ml^{-1}, and **f** 200 µg ml^{-1} of *H. laurifolia* seeds ethyl acetate extract on K562 cells

Z,*Z*-3,11-octadecadien-1-ol acetate; β-sitosterol glucoside (sitogluside); Lignoceric acid; hexadecanoic acid (palmitic acid); 1-heptanol; luteolin; and 9,12-octadecadien-1-ol, (Z,Z) in *H. laurifolia* seed ethyl acetate extract. Sini et al. (2005a) analyzed the fatty acid-rich portion of *H. laurifolia* by GC–MS and showed the presence of chaulmoogric acid, gorlic acid, hydnocarpic acid, lignoceric acid, oleic acid, stearic acid, and palmitic acid which was also comparable to the present study. In the present study, cyclobutylamine; methyl thioacetate (ethanethioic acid); *Z*,*Z*-3,11-octadecadien-1-ol acetate; 1-heptanol; and 9,12-octadecadien-1-ol, (*Z*,*Z*) were identified for the first time in *H. laurifolia* seed ethyl acetate extract. Damle et al. (1978) reported that chaulmoogric acid showed significant anti-mycobacterium activity against *Mycobacterium intracellulare*. Levy (1975) confirmed the anti-mycobacterium activity of chaulmoogric and hydnocarpic acid against *M. leprae*. The β-sitosterol glucoside is a phytosterol that has been reported as a bioactive molecule, which was known for anti-inflammatory, immunomodulatory, and anti-cancer activities as well as controlling blood glucose and reducing cholesterol (López-Salazar et al. 2019). Hexadecanoic acid usually presents in various plant natural oils/fats and is also found in *H. laurifolia* seeds. It exhibited potential anticancer, antioxidant activities (Kim et al. 2020), and antimicrobial efficacy against various human pathogens (Sagwan et al. 2012). Several studies reported that luteolin has numerous pharmacological properties including anti-inflammatory, anticancer, antioxidant, antimicrobial, and anti-allergic (Wang and Xie 2010). The presence of all these biologically active compounds confirms the huge

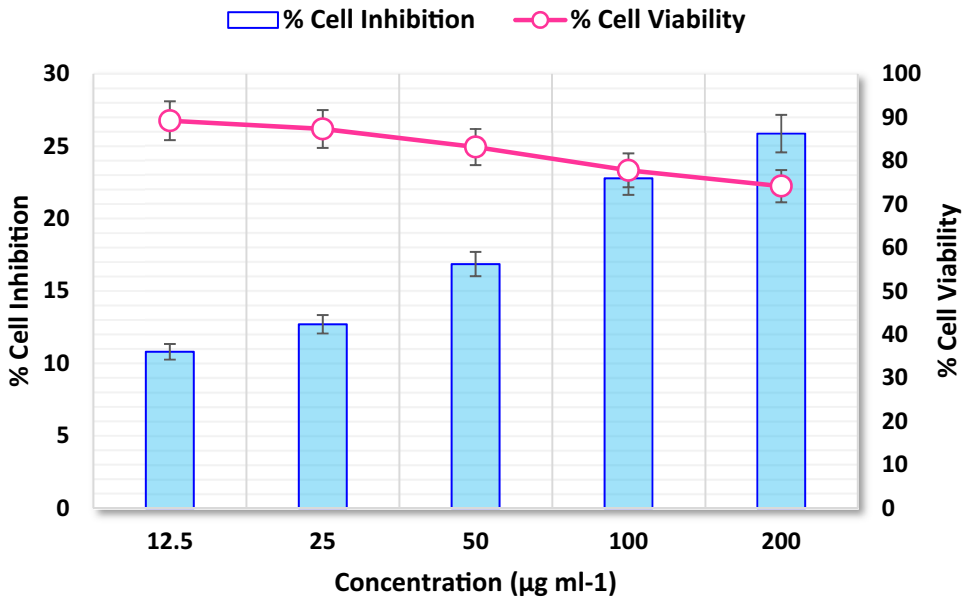


Fig. 11 Percent cell inhibition and viability of PBMCs by *H. laurifolia* seeds ethyl acetate extract via MTT bioassay

pharmacological and therapeutic properties of ethyl acetate seed extract of *H. laurifolia.*

Total phenolic and flavonoid contents are correlated with the defensive antioxidant capacity of the extract because phenolics are proved to be very strong antioxidants. Several studies done all over the world have proved that phenolic compounds are having exceptional antioxidant as well as antimicrobial potential (Sharma et al. 2021; Zhou et al. 2020). Yuvaraja et al. 2018 analyzed TPC and TFC of the chloroform seed extract of *H. laurifolia* and reported 70 ± 14 mg GAE g^{-1} and 185 ± 18 RTNE g^{-1} respectively that showed high phenolic as well as flavonoid content that is also reported in the present research work. Thus, *H. laurifolia* seeds ethyl acetate extract contains a good amount of phenolics and flavonoid compounds which implicated its usefulness as a good antioxidant agent.

Free radicals like reactive oxygen species (ROS) cause numerous disorders and antioxidant compounds can counteract them either by scavenging ROS or by channelizing antioxidant defense mechanisms. Compounds having antioxidant capacity minimize the harmful effects cause by free radicals before any kind of cell, enzymes, carbohydrates, lipids, proteins, and DNA damage. Herbal products have a wide range of biologically active phytocompounds serving as antioxidants with no side effects as compared to synthetic drugs (Ilavarasan et al. 2005). In the present study, *H. laurifolia* seeds ethyl acetate extract possessed very good antioxidant activity with a very low IC_{50} value of 10.64 ± 0.48 µg ml^{-1} in DPPH assay and 7.81 µg ml^{-1} in ABTS assay. The reason for such an excellent radical scavenging activity is metabolically active phytoconstituents as phenolic compounds that are present in the *H. laurifolia* seeds ethyl acetate extract have the potential of donating a hydrogen atom to free radical to stabilize them and prevent potential damage. The low IC_{50} value supports the significance of *H. laurifolia* seeds as a promising natural antioxidant agent, hence can be used in the pharmaceutical as well as nutritional industries. Yuvaraja et al. (2020), reported the free radical scavenging potential of *H. laurifolia* ethyl acetate seed extract by DPPH as well as ABTS assay and reported the IC_{50} value 23.03 µg ml^{-1} and 15.51 µg ml^{-1} respectively, showing the potential antioxidant activity of ethyl acetate extract. In the present study, the IC_{50} values in both the assays were observed slightly lower than that of the previous study this might be due to the difference in seed samples and extraction process.

Phytochemical screening of *H. pentandra* (Syn. *H. laurifolia*) extracts revealed the presence of good amounts of phenolics, it makes *H. pentandra* a potent antioxidant medicinal plant. Shyam et al. 2013 reported excellent antioxidant activity of the *H. pentandra* seeds ethyl acetate extract that was comparable to the present investigation. Reddy et al. (2005), also investigated the antioxidant activity of *H. wightiana* (Syn. *H. laurifolia*) seed extracts and confirmed that acetone extract possesses strong DPPH as well as ABTS radical scavenging efficacy with the IC_{50} value of 32.54 µg ml^{-1}.

The present study reported *H. laurifolia* seed ethyl acetate extract as a promising antimicrobial as well as an antibiofilm agent. In addition, MIC, MBC/MFC data showed that ethyl acetate extract inhibited and eradicated the growth of pathogens with very small concentrations that indicate the efficacy of biologically active phytoconstituents present in it. Very little literature is available on the antimicrobial potential of *H. laurifolia* but, an earlier study demonstrated that seed extracts of *H. wightiana* (Syn. *H. laurifolia*) exerted potential antimicrobial efficacy against *Salmonella typhi,*

Escherichia coli, Klebsiella pneumoniae, and *Proteus mirabilis* (Samuel et al. 2010). Kekuda et al. (2017) investigated the antimicrobial activity of *H. pentandra* leaves against *S. epidermidis, S. aureus, B. subtilis, E. coli, P. aeruginosa, B. cereus, S. typhimurium,* and antifungal activity against *Curvularia* sp., *Alternaria* sp., *Fusarium* sp. and reported that leaf extract inhibits all the test pathogens. The present investigation reported that the growth of *P. aeruginosa* was ceased within 6–8 h and further growth was not observed on the exposure of 1 × MIC and 2 × MIC concentration of extract, which directly indicated prolonged antimicrobial efficacy of *H. laurifolia* seeds. The time-killing profile provides the understanding to evaluate the pharmacodynamic properties of an antimicrobial compound by estimating microbial growth inhibition as a function of dose and time intervals (Rajput et al. 2021).

The biofilm formation is a factor of virulence in bacteria that protect them from the shear forces, immune system, and antimicrobial drugs. Biofilms are associated with various pathogenic manifestations and help pathogens to survive in the host during etiology and treatment (Landini et al. 2010). The present study reported the antibiofilm efficacy of *H. laurifolia* seed ethyl acetate extract for the first time against *P. aeruginosa* and the data present the potential antibiofilm activity. The LM/FE-SEM micrographs of extract-treated biofilms showed concentration-dependent antibiofilm activity on *P. aeruginosa* biofilm cells. FE-SEM micrographs displayed that on the treatment of ½ × MIC and 1 × MIC, the bacterial biofilm collapsed with drastic changes in the cell wall morphology such as immense blebbing, invaginations, disorganization, and finally, complete cells lysis. Gill and Holley (2006), proposed that on the treatment of extract on biofilms, the phytochemicals interact with the proteins, and enzymes of the cell membrane and disrupt the proton flux outside the cell that further leads cell death. Latha et al. (2010); Nithyanand et al. (2015) also suggested the concertation-dependent efficacy of extract on biofilm as well as planktonic cells.

Medicinal plants have unlimited capacity to generate bioactive phytochemicals that fascinate researchers in the quest for novel chemotherapeutics and the persistent search for such anticancer compounds may be a promising strategy for cancer prevention. Such bioactive compounds hold great potential as drug candidates due to their low toxicity, safety, and wide acceptance among the public. Therefore, in the present research work, the *H. laurifolia* seed ethyl acetate extract was evaluated as a new anticancer agent via MTT assay. The data of in vitro assay displayed that on the exposure of lower concentrations of *H. laurifolia* seed ethyl acetate extract, there was a mild cytotoxicity effect on the K562 cells, whereas, at higher concentrations, comparatively remarkable cytotoxicity was observed. National Cancer Institute (NCI), USA, set the limit of cytotoxic effect for crude extracts of medicinal plants which is an IC_{50} value of less than 30 µg ml^{-1} after the exposure of 72 h in a preliminary assay (Ogbole et al. 2017). The present study showed a potent cytotoxic effect of ethyl acetate extract of *H. laurifolia* on K562 cancer cells with an IC_{50} (25.41 µg ml^{-1}) which was lower than that of stated by NCI norms for anticancer agents. On the other hand, it is interesting to report that *H. laurifolia* seed ethyl acetate extract did not show potential cytotoxicity on normal PBMCs, suggesting it is a safe, selective, and effective anticancer agent with an SI value of 18.99 which is more toxic to cancer cells than normal cells. The heterogeneity, biochemical, genetic, and molecular differences in the normal and cancerous cells might be the reason behind the selective toxicity (McLachlan et al. 2005). However, further investigations are needed on the molecular pathways and the mechanism of action to gain an insight into *H. laurifolia* seed ethyl acetate extract on the cell death pathway of K562 cells.

The present piece of work is clinically important and innovative as it demonstrated the efficacy of *H. laurifolia* seeds ethyl acetate extract as a remarkable antimicrobial, antibiofilm, anticancer, and antioxidant agent. To the best of our knowledge, the present piece of work was the first report on the biofilm inhibitory efficacy evaluation of *H. laurifolia* seed ethyl acetate extract against *P. aeruginosa*. Furthermore, the anticancer activity was also reported for the first time in the present investigation.

Conclusion

The present data conclusively validate the ethnopharmacological properties (antioxidant, antimicrobial, antibiofilm, and anticancer activities) of phytochemicals present in *H. laurifolia* seed ethyl acetate extract. It possesses high TPC and TFC values which correspond to its remarkable antioxidative activity. It is a potent antimicrobial agent against all the human pathogenic bacteria and fungi, being superior to commercial drugs. It can effectively inhibit the *P. aeruginosa* biofilm formation, therefore, reducing the virulence of the pathogen. Furthermore, it is a potent anticancer agent too as it displayed selective toxicity on K562 cells and relatively very low cytotoxicity on normal PBMCs. The future perspective of the study is to isolate and identify the biologically active phytocompounds from the extract and the evaluation of its molecular mechanism of action for various potential activities such as antimicrobial, antioxidant, antibiofilm, and anticancer.

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Declarations

Conflict of interest The authors have no conflict of interest to disclose.

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