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Research Article

In vitro evaluation of antimicrobial and anticancer potential of *Artemisia absinthium* growing in Kashmir Himalayas

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Abstract
Herbal medicines are an important and growing part of International pharmacopeia. Research and testing enhance our understanding of their medical properties, making them a safer alternative or preferable option to allopathic medication. Plant-derived pharmaceuticals are gaining popularity due to the belief that "green medicine" is safer and more trustworthy than expensive synthetic drugs. This study aimed to evaluate the antimicrobial and anticancer potential of the methanolic leaf extract of *Artemisia absinthium* against human lung cancer A549 cell line by well diffusion method and MTT assay, respectively. The *A. absinthium* leaf extract showed the highest activity against *Enterococcus faecalis* (20 ± 0.7 mm), and *Escherichia coli* (18 ± 0.8 mm), followed by *Pseudomonas aeruginosa* (16 ± 0.6 mm), *Candida albicans* (14 ± 0.9 mm) and *Staphylococcus aureus* (13 ± 0.8 mm), with MIC values 128, 128, 128, 256 and 256 µg/mL respectively. The methanolic extract of *A. absinthium* showed significant ($p \leq 0.05$) cytotoxicity against the A549 cancer cell line with an IC_{50} value of 36.8 µg/mL. The present study's findings give strong evidence for using the methanolic leaf extract of *A. absinthium* as an effective ethnomedicinal agent and a possible candidate for treating various human diseases and a potent bioactive agent in anticancer medications.

Keywords: *A. absinthium*, Antimicrobial activity, cytotoxic activity, Kashmir Himalayas, Medicinal plants

INTRODUCTION

Plants have played an important role in sustaining and improving the quality of human life for millennia. Furthermore, plants were and continue to be a rich source of innovative therapeutic agents for treating a wide range of primary health care illnesses. Numerous studies demonstrate that natural compounds can treat complicated disorders (Newman and Cragg, 2020; Zaki *et al.,* 2021; Lautie *et al.,* 2020). In cancer therapy, medicinal plants contribute to anticancer medications that limit tumor progression without any adverse effects (Iqbal *et al.,* 2017; Huang *et al.,* 2021). As a result, plants are still being studied worldwide for their anticancer potential.
Additionally, microbial infections are seen as a worldwide danger. Despite the fact that pharmaceutical corporations have released several novel antibiotics, microbial resistance has grown (Murray *et al.,* 2022). As a result, more attention is being paid to medicinal plants as a possible source of novel antimicrobials (Vaou *et al.,* 2021).

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Artemisia absinthium L (Asteraceae) is a well-known medicinal herb commonly known as wormwood. It is native to Eurasia and North Africa. In India, it is found in Kashmir Valley. Traditionally *A. absinthium* has been used to treat gastritis, gastric pain, indigestion, splenomegaly, hepatomegaly and hepatitis. It has also been documented to have antihelmintic, anticarcinogenic, neuroprotective, analgesic, hepatoprotective and antidepressant activity (Sofi *et al.,* 2022; Szopa *et al.,* 2020; Ahamad, 2019; Jahangir *et al.,* 2019). Therefore, the present study reports the therapeutic validation of the *A. absinthium* plant, particularly with antimicrobial and anticancer effects.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (USA). Fetal Bovine Serum(FBS), 0.25 % trypsin–EDTA and Antibiotic Antimycotic Solution (100×) with 10,000 units Penicillin, 10 mg streptomycin and 25 µg/mL amphotericin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). While 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was purchased from Sisco Research Laboratories Pvt Ltd (SRL chemicals), India. All consumables and cell culture wares, including tissue culture flasks and 96 well plate were purchased from Tarsons, India. Mueller Hinton Agar (MHA) and methanol were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai). The rest of the chemicals were procured locally of cell culture grade.

Fig. 1. *Artemisia absinthium plant used for the present study*

Collection of Plant material

The *Art*emisia *absinthium* plant was collected from the Daksum area of Anantnag, Jammu and Kashmir, India, at an altitude of 2438 meters above sea level (Figs. 1 and 2). The identification and authenticity process of the plant was completed in the Kashmir University (CBT-botany) vide voucher specimen number 2837-(KASH) Herbarium. The leaves of the plant were shade dried in hygienic conditions for at least fifteen days. After that, the leaves were crushed into a coarse powder in an electrical grinder machine and packaged carefully for further processing.

Extraction process

A simple maceration process was employed for the extraction of the plant material. 10 g of coarse powder

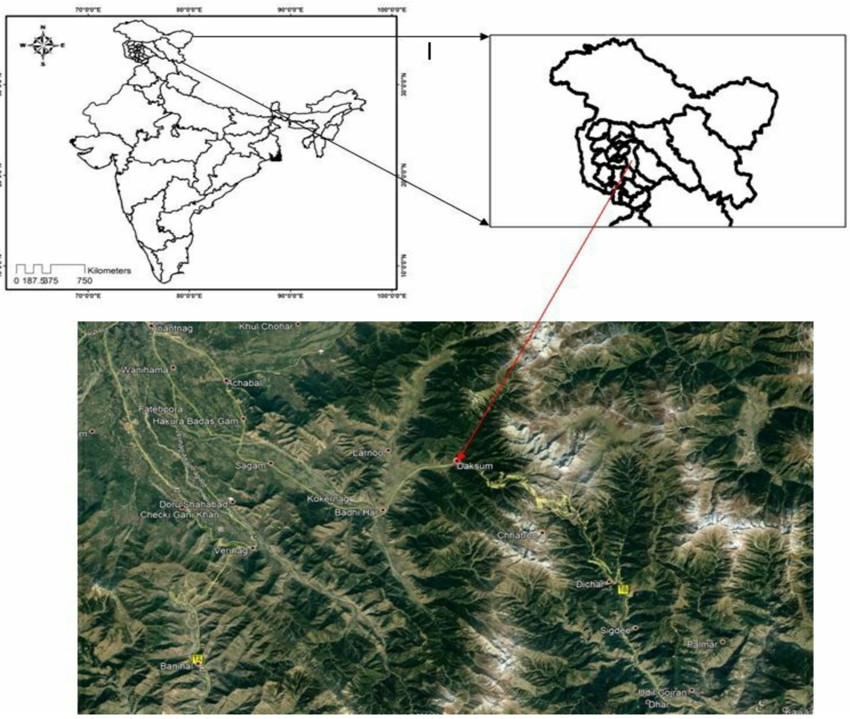


Fig. 2. *Collection site Daksum Anantnag Jammu and Kashmir, India*

was mixed with 100 mL of methanol in a 250 ml flask and the mixture was kept in a shaker for 24 h. This was followed by filtration of the reaction mixture using Whatman filter paper No.1. The filtrate collected was left for evaporation of the solvent to obtain a concentrated mass. The procedure was repeated three times to get the desired quantity and quality of the sample for further analysis **(**Ben *et al.,* 2018)**.**

Antimicrobial activity

The extract was evaluated for antimicrobial activity against gram-positive *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212; gram-negative strains *Pseudomonas aeruginosa* ATCC 15442 and *Escherichia coli* ATCC 11229; and Fungus *Candida albicans* ATCC 10231 using a well diffusion method.

Mueller Hinton Agar (MHA) was prepared and poured into sterile Petri plates. These nutrient agar plates were inoculated within 24 h. bacterial suspension by swabbing sticks to produce a lawn of bacterial growth. Wells of 6mm were cut with the help of a sterile cork-borer and different micro volumes, 25 µL, 50 µL, 75 µL, and 100 µL from 10 mg/mL stock solution were added to newly-created wells. Amphilox, Ciprofloxacin and Fluconazole were used as a positive control for gram positive, gram-negative bacteria and fungus, respectively. Dimethyl Sulfoxide (DMSO) was used as a negative control. The zone of inhibition was calculated in mm. The minimum inhibitory concentration (MIC) value in µg/mL was determined using the broth dilution method in 96 well plates described below. The results were calculated after 24 h. of incubation.

Determination of the MIC by dilution technique method

The experiment was conducted in flat bottomed 96 well plate using the methods described by (Gabrielson *et al*., 2002). First, 5µL of 12 hour old test pathogens were introduced to 10 wells containing 100 µL of serially diluted concentrations of *Art*emisia *absinthium* leaf extract (512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg/mL). The plates were incubated at 37°C for 24 h, followed by adding 10 µL of freshly prepared MTT (5 mg/mL) to all wells. After 2 h. of incubation at 37°C, 100 µL of DMSO (Dimethyl Sulfoxide) solution was applied as the solubilizing agent. The color transition was visually observed to determine MIC.

Cell culture

Human lung cancer cell line A549 procured from King Institute of Preventive Medicine & Research Guindy, Chennai, Tamil Nadu, India, was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution, maintained throughout in a humidified incubator with a continuous supply of 5% CO_2 and a constant temperature of 37 °C. On reaching 70-80% confluency, cells were trypsinized (1x trypsin-EDTA solution) and further seeded into a 96 well plates. After incubating the cells for 21-24 h, the cells attached to the surface and attained normal morphology. The cells were then used for further experiments (Ashma *et al.,* 2022).

Extract preparation

Methanolic leaf extract of *Art*emisia *absinthium* was prepared in Dulbecco's Modified Eagle Medium (DMEM) with a stock concentration of 1 mg/mL for further use in cell viability assay.

Cell viability assay

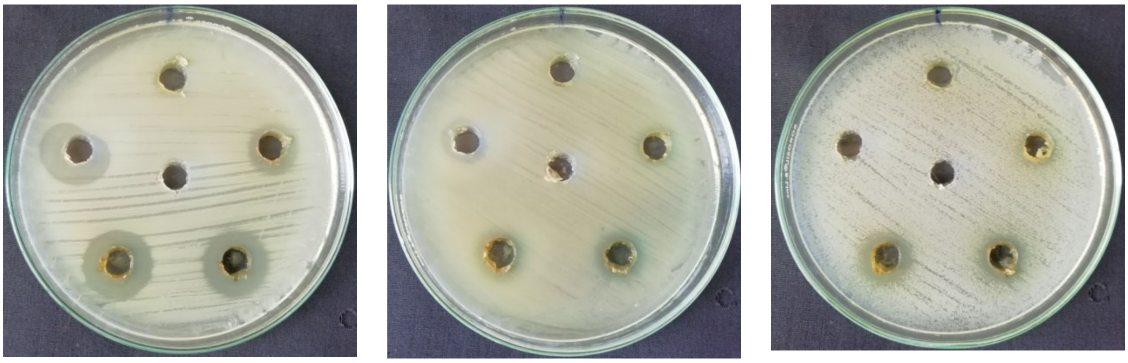
Cultured A549 cells were seeded into a flat bottom 96 well plate at a density of approximately 6×10^3/well. Complete media (10%Fetal Bovine Serum in Dulbecco's Modified Eagle Medium) was replaced with 3% Fetal Bovine Serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) medium. The cells were treated with *A. absinthium* methanolic extract at different concentrations ranging from 5-100 µg/mL and incubated for 24 hours. Media containing drug extract was aspirated gently and replaced with a fresh serum-free medium. 10 µL of MTT reagent (5 mg/mL) was added to each well in the dark and incubated for 3-4 h. Subsequently, the MTT reagent was discarded and 100 µL of Dimethyl Sulfoxide (DMSO) was added to each well and purple-colored formazan crystals were formed. The reaction mixture was briefly agitated on an orbital plate shaker to dissolve formazan crystals, and absorbance was measured at 590 nm (with an iMark™ microplate reader (Bio-Rad). Percentage cell viability was calculated using the following formula.

% of viability =Absorbance of treated cells with *A. absinthium* leaf extract /Absorbance of control cells ×100 Eq. 1

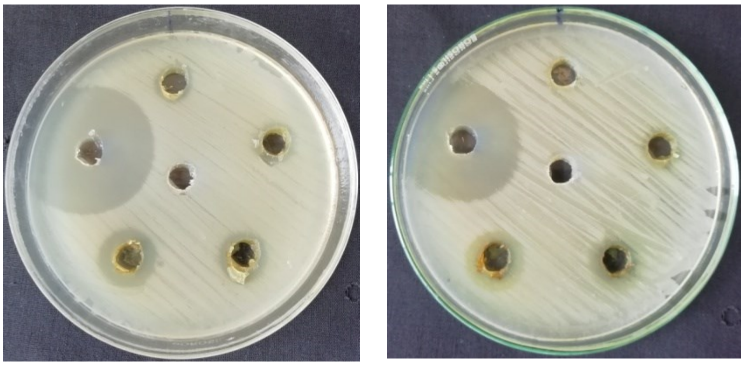
RESULTS

Antimicrobial activity of *A. absinthium*

The *A. absinthium* medicinal plant, which is utilized for different remedies by local populations, was also tested against ATCC microbial cultures. The crude methanol extract of *A. absinthium* showed antimicrobial activity against all investigated microbial strains. The highest activity was conferred against *Enterococcus faecalis* (20 ± 0.7 mm), *Escherichia coli* (18 ± 0.8mm) and *Pseudomonas aeruginosa* (16 ± 0.6 mm), followed by *Candida albicans* (14±0.9 mm) and *Staphylococcus aureus* (13 ± 0.8 mm), with MIC values 128, 128, 128, 256 and 256 µg/mL respectively (Table 2). However, the standard drug did not show any activity against the *C. albicans,* as shown in Fig.3 and Table 1.



E. faecalis *S. aureus* *C. albicans*



E. coli *P. aeruginosa*

Fig. 3. *Showing antimicrobial activity of A. absinthium methanolic leaf extract using well diffusion method. (Numerals depicted on each petri plate in the Fig. 4 represent the wells introduced with different micro volumes of A. absinthium leaf extract (10 mg/mL stock solution), standard drugs 25μl (1 mg/ mL) and negative control 1: 25 μl; 2: 50 μl; 3: 75 μl; 4: 100 μl; 5: 25μl Standard drug positive control,Ampilox for Gram-positive: E. faecalis and S. aureus, Ciprofloxacin for Gram-negative :P. aeruginosa and, E. coli, Fluconazole for fungi: C. albicans. 6: DMSO as a negative control).*

Anticancerous activity of *A. absinthium*.

The typical cytotoxicity of methanolic leaf extract of *A. absinthium* towards A549 was found to be significant. At the end of the experiment, the IC50 value of *A. absinthium* methanolic extract was found to be 36.8 μg/ mL (Fig 4). Moreover, an increase in cytotoxicity was observed with and an increase in the concentration of the *A. absinthium* extract. Based on the MTT results, it was found that the methanolic extract of *A. absinthium* exhibited significant anticancer activity against the A549 lung cancer cell line in a dose-dependent manner. The current study suggests that *A. absinthium* leaves methanolic extract has significant anticancer activity, indicating its potential use in cancer prevention and chemotherapy.

DISCUSSION

The alarming rise in the incidence of bacterial infections is presently posing a serious threat to global public health. Antibiotic resistance, and the emergence of new pathogens with the potential of rapid worldwide transmission, exacerbates the situation, fueling the hunt for new bioactive agents. There are undoubtedly many drugs currently available today to treat bacterial infections. Still, unfortunately, they all have substantial adverse side effects, limiting their usage in specific sectors of the population. As a result, there is an ongoing and pressing need to develop novel antibacterial agents with a high safety index (Aslam *et al.,* 2018; Miethke *et al.,* 2021). Taking into account the rising need for novel remedies to combat various infections caused by antibiotic resistant microbes, Artemisia L. species can act as the promising raw material for their creation. In addition, the biologically active compounds of *Artemisia* L. can neutralize individual determinants of antibiotic resistance and thereby restore the susceptibility of resistant strains to the corresponding drugs (Hrytsyk *et al.,* 2021; .Liu *et al*., 1992; Li *et al*., 2011; Cremer *et al.,* 2015), impede the process of obtaining the antibiotic resistance (Dülger *et al.,* 1999), inhibit the formation of microbial biofilms (Hrytsyk *et al.,* 2021; Liu *et al.,* 1992; Pandey *et al.,* 2017). Organic solvents such as methanol, ethanol, hexane and acetone are often used to extract bioactive compounds since these solvents easily elute most polar molecules (Abubakar *et al.,* 2020).

Table 1. Antimicrobial activity of the *A. absinthium* methanolic leaf extract against human pathogens

S. No	Pathogen Name	Zone of inhibition (mm)					
		25 µL	50 µL	75 µL	100 µL	Standard drug	Negative control
1	*E. faecalis*	-	14 ± 0.8	19 ± 0.5	20 ± 0.7	18 ± 0.6	-
2	*S. aureus*	-	-	12 ± 0.7	13 ± 0.8	13 ± 0.9	-
3	*C. albicans*	-	11 ± 0.8	13 ± 0.6	14 ± 0.9	-	-
4	*E. coli*	11 ± 0.9	12 ± 0.5	14 ± 0.4	18 ± 0.8	36 ± 0.9	-
5	*P.aeruginosa*	-	-	12 ± 0.5	16 ± 0.6	31 ± 0.5	-

(-) means no activity. The value is the mean± standard deviation of three repetitions (n= 3)

Table 2. Minimum inhibitory concentration of *A. absinthium* methanolic leaf extract against human pathogens

Minimum inhibitory concentration (MIC) (µg/ml)				
Gram-positive bacterium		**Gram-negative bacterium**		**Fungal pathogens**
S. aureus	***E. faecalis***	***E. coli***	***P. aeruginosa.***	***C. albicans***
256	128	128	128	256

Many studies have addressed the antimicrobial potential of plants from the *Artemisia* genus (Ahameethunisa and Hopper, 2010). *Artemisia annua* and *Artemisia afra* extracts exhibited strong bactericidal activity against *Mycobacterium Tuberculosis* (Martini *et al.,* 2020). In another study by Khan *et al.,* 2018, ethanolic extracts of *A. absinthium* exhibited a dose-dependent antibacterial activity exclusively against *S. aureus* and *E. faecium* with an MIC_{50} value of 256 µg/mL. A subsequent study was done by (Habibipour *et al.,* 2015) in which a hydro-alcoholic extract from the A. absinthium was active against *S. aureus, P. aeruginosa*, *Bacillus subtilis*, *Haemophilus influenza* and *Bacillus cereus* with a dose of 750 mg/mL of the extract. Reports have shown that *A. absinthium* showed antimicrobial activity. Ethanolic extract of *A. absinthium* inhibits *S. aureus* ATCC (29213) with a zone of inhibition 10 -15 mm, (Dülger *et al.,* 1999), which is consistent with our results but had no potent antimicrobial effects against *E. coli*, *E. faecalis,* which is in contrast to our studies. In the present study, methanolic extract showed antimicrobial activity against all tested pathogens. The highest zone of inhibition (20 ± 0.7mm) was seen in *E. faecalis*. This might be due to the difference in the extraction solvent, geographical location and bacterial strain used. *A. absinthium* has also been reported to have antifungal activity. The study by (Joshi *et al.,* 2013) stated that the essential oil of *A. Absinthium* was effective against *Micrococcus leutus*. Recently, the silver nanoparticles synthesized using the aqueous extract of *A. absinthium* has shown potent antifungal activity against some pathogens of *Candida* species (Del *et al*., 2019). In our study, methanolic extract of *A. absinthium* was also effective against *C. albicans* with (14 ± 0.9 mm) inhibition zone. Meanwhile, the standard drugs did not show any activity against C*. albicans*. This shows that *A. absinthium* leaves have potent activity against all tested pathogens

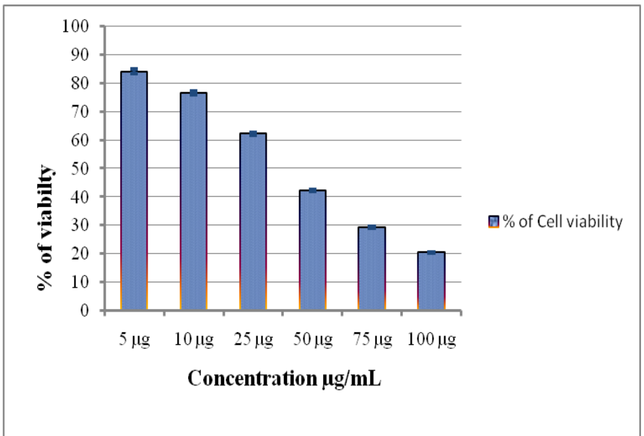


Fig. 4. *MTT assay of A. absinthium methanolic leaf extract on A549 Cell line (Bar graphs represent mean of three absorbance values (n=3) and error bars represent ± SD from the mean)*

Plants have unlimited potential to produce substances that entice researchers to hunt for novel chemotherapeutics (Dehelean *et al.,* 2021). A realistic and promising strategy for cancer prevention is the continuous search for new anticancer agents in plant medicines (Garcia-Oliveira *et al*., 2021). Plant-derived natural compounds with anticancer effects include terpenoids, alkaloids, and phenylpropanoids (Desam *et al*., 2022). Natural product research can lead to the discovery of a plethora of novel chemical structures with a wide range of biological functions (Amit & Singh, (2022), Iqbal *et al.,* 2017; Rajput *et al.,* 2021). Over 60% of the anticancer drugs in clinical use today are either obtained from natural products or are based on natural product templates (Cragg and Pezzuto, 2016). Since cancer death rates are so high, finding new treatments is a top priority. The genus *Artemisia* is a rich source of anti-tumor compounds such as terpenoids, sesquiterpene lactones and flavonoids (Nigam *et al.,* 2019). Previous studies have shown that *A. absinthium* extract has strong anti-proliferative effects against human breast cancer cells (Shafi *et al.,* 2012). The present research explores the effects of methanolic leaf extract against human lung cancer A549 cells. The findings obtained from the MTT assay exhibited that *A. absinthium* methanolic extract significantly ($p \leq 0.05$) inhibited the growth of A549 cancer cells with an IC_{50} of 36.8 μg/mL for 24 h, indicating a promising anti-lung cancer activity of methanolic extract of *A. absinthium* leaf extract. As can be seen in (Fig.4) MTT assay showed that methanolic extract could inhibit the growth of A549 cancer cells in a dose-dependent manner. Shafi *et al.* (2012) observed that *A. absinthium* had considerable cytotoxic activities against MDA-MB-231 and MCF-7 cells with IC_{50} values of 25 μg/mL and 20 μg/mL, respectively. Further, they reported that it induced apoptosis in both cells.

Our results support earlier findings by Gordanian *et al*., 2014, who found that extracts of *A. absinthium* and *A. vulgaris* had higher anticancer potential against the HEK-293 AND MCF7 cell lines than did extracts of *A. incana, A. spicigera,* and *A. fragrans.* Another study by Lian *et al.* (2018) revealed that the methanolic extract of *A. vulgaris* has considerable anti-proliferative effects against human colon cancer cells (HCT-15) with an IC_{50} value of 50 μg/mL. An in-depth investigation would help in better understanding and development of future therapeutics. Isolation of bioactive compounds and in vivo studies would reveal the true potential of the extract against different cancers and pathogenic microorganisms.

Conclusion

The present study concluded that the methanolic extract of *A. absinthium* significantly ($p \leq 0.05$) inhibited all the tested pathogens and induced characteristic cell death in lung (A549) cell lines. The effective bioactive potential of this plant provides the basis for a new natural source on the drug ability list of pharmacognosy platforms.

Conflict of interest

The authors declare that they have no conflict of interest.

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