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RESEARCH ARTICLE

BHANG ESSENTIAL OIL POSSESSES ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

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Abstract: The present study evaluated the antioxidant and antibacterial activity of essential oil extracted from fresh leaves of *Cannabis sativa.* Antioxidant potential was determined using DPPH radical scavenging, ferric reducing antioxidant power (FRAP) assay, while antibacterial efficacy was assessed by the agar well diffusion method against gram-positive and gram-negative bacterial strains. The essential oil of the plant exhibited significant antioxidant potential, as evidenced by its free radical scavenging ability and ferric ion reducing capacity and also inhibited the growth of all tested bacterial strains, with the highest antibacterial activity observed against *Escherichia coli*, followed by *Micrococcus luteus.* The findings indicate that *C. sativa* essential oil possesses promising antioxidant and antibacterial properties, which may be attributed to the synergistic action of its volatile phytoconstituents. The results highlight its potential for future applications as a natural bioactive ingredient in pharmaceutical, nutraceutical and food preservation systems.

Keywords: *Cannabis sativa*, Essential oil, DPPH, FRAP, Antibacterial

INTRODUCTION

Cannabis sativa, commonly known as bhang or Indian hemp, is an annual herbaceous plant belonging to the family Cannabaceae, native to Central and South Asia and widely used in traditional medicine for the treatment of pain, inflammation, rheumatism, anxiety and several neurological disorders (Bonini *et al*., 2018). The plant has gained considerable scientific attention owing to its essential oil, which is rich in volatile bioactive constituents possessing diverse pharmacological properties (Pollastro *et al*., 2018). The essential oil is composed predominantly of mono- and sesquiterpenes, with β-caryophyllene, α-humulene, caryophyllene oxide, α-pinene, β-pinene, myrcene, limonene and terpinolene being the major constituents (Novak *et al*., 2001; Zheljazkov *et al*., 2020). These volatile compounds are largely responsible for the characteristic aroma of the plant and contribute significantly to its antioxidant, antimicrobial and anti-inflammatory activities (El-Mernissi *et al*., 2024).

The essential oil of *Cannabis sativa* is characterized by a complex mixture of volatile compounds, accounting for approximately 99.8% of the total oil composition, belonging to various chemical classes, including cannabinoids, terpenoids, flavonoids, alkaloids, steroids, fatty acids and amino acids (Pollastro *et al*., 2018). The oil is composed mainly of monoterpene hydrocarbons (54.0%) and sesquiterpene hydrocarbons (44.2%), with β-caryophyllene, α-humulene, caryophyllene oxide, α-pinene, β-pinene, myrcene, limonene and terpinolene reported as the major constituents. These volatile compounds are responsible for the characteristic aroma of the essential oil and contribute to its biological activities. However, the composition of essential oil varies among cultivars due to differences in genetic makeup and environmental conditions (Novak *et al*., 2001; Zheljazkov *et al*., 2020). The antioxidant potential of *Cannabis sativa* essential oil has been widely attributed to its terpene-rich composition, particularly sesquiterpenes and monoterpenes, which are capable of neutralizing reactive oxygen species and preventing oxidative damage. Experimental studies have demonstrated that the essential oil exhibits dose-dependent free radical scavenging activity together with appreciable ferric ion reducing capacity, indicating its ability to donate hydrogen atoms and electrons to unstable free radicals (Palmieri *et al*., 2021). The essential oil of the plant exert its antibacterial effects primarily by interacting with the phospholipid bilayer of bacterial cell membranes, leading to disruption of membrane integrity, interfere with membrane-bound enzymes involved in respiration and ATP synthesis, thereby impairing bacterial metabolism and inhibiting cell growth.

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MATERIALS AND METHODS

Extraction of essential oil: Bhang leaves were collected from the herbal garden of SKUAST-Jammu, Chatha, in the month of July, 2025. Collected leaves were washed thoroughly. Essential oil from fresh leaves was extracted by hydrodistillation using clevenger-type apparatus (Guleria *et al.,* 2008). The leaves were repeatedly subjected to hydrodistillation until no more essential oil was obtained. The obtained oil was collected, dehydrated with anhydrous sodium sulphate and was stored in glass vials at -4°C for further analysis. The weight and amount of oil was determined in order to calculate the percent yield (Fig. 1).The percent yield of essential oil was determined using the following formula:

$$\text{Percent yield} = \frac{\text{Weight of oil collected (in grams)} \times 100}{\text{100 grams of leaf sample}}$$

Determination of antioxidant activity:

The antioxidant activity of the essential oil was evaluated by measuring the ability to scavenge DPPH radicals (Bozin *et al*., 2006). Different concentrations of the essential oil were taken from 10 mg/ml stock solution prepared in methanol and volume was made to 1 ml with methanol. After that 1 ml of the 90 µM DPPH solution (prepared in methanol) and then 2 ml methanol was added to make the final volume of the reaction mixture upto 4ml. The final reaction mixture was shaken well and kept in the dark for 1 hour at room temperature. After incubation, absorbance was taken at 517 nm wavelength by using double beam UV–vis spectrophotometer. Methanol was taken as the negative control whereas the reaction mixture without essential oil was taken as the positive control.

Percentage of radical scavenging activity (% RSA) = $(1\text{-}A_{sample}/A_{blank}) \times 100$

Where, A_{blank} is the absorbance of the control (containing all the reagents except sample) and A_{sample} is the absorbance of the test sample. Absorbance decreases with increase in antioxidant activity. The concentration of the essential oil at which absorbance becomes exactly half of that of the control is known as IC_{50}.Butylated hydroxytoluene (BHT) was used as the reference compound.

The FRAP assay was conducted according to Benzie and Strain (1996). The working FRAP reagent was freshly prepared by mixing 100 ml of sodium acetate buffer (300 mM, pH 3.6), 10ml of 10 mM TPTZ [2,4,6-tri (2-Pyridyl)-S-triazine] solution in 40mM HCl and 10ml of 20 mM $FeCl_3$and 12ml distilled water. To carry out the assay, different concentrations of essential oil was mixed with 3 ml of FRAP reagent. After incubation for 10 min, the absorbance was measured at 593 nm. The FRAP activity was calculated from the calibration curve of ferrous sulphate ($FeSO_4.2H_2O$) and the value of FRAP was expressed as mM $FeSO_4.2H_2O$ equivalents/g of dry weight of the essential oil.

Determination of antibacterial activity:

Bacterial strains used in the present study: The study utilized three-gram positive bacterial strains namely *Bacillus subtilis* MTCC 2389, *Staphylococcus aureus* MTCC 7443 and *Micrococcus luteus* MTCC 4821, as well as two-gram negative strains, *Escherichia coli* MTCC2127 and *Klebsiella pneumoniae* MTCC7172. These strains were procured from IMTECH Chandigarh. The bacterial cultures were preserved in nutrient agar slants and stored at -4^0C. These cultures were used as stock cultures.

Preparation of inoculums: The selected bacterial strains were introduced into a nutrient broth and incubated for 24 hours at a temperature of 37 ^{0}C. The optical density of the resulting active bacterial cultures was adjusted to match the 0.5 Macfarland standard. This was achieved by diluting the cultures with nutrient broth. The turbidity of the cultures was then determined by measuring the absorbance at 600 nm using a spectrophotometer.

Agar-well diffusion method: The antimicrobial activity of the essential oil was evaluated qualitatively using the agar - well diffusion method, as described by Feyza *et al*., 2009, with some modifications. The sterilized nutrient agar (20 ml) was poured into sterile petri plates and allowed to solidify undisturbed in a sterile laminar air flow chamber. Once solidified, the petri plates were uniformly inoculated with 100 µl of a bacterial suspension (10^9 CFU / ml) using a sterile glass spreader. The plates were then left undisturbed for a few minutes to allow the bacterial suspension to be absorbed into the nutrient agar. Wells with a diameter of 6 mm were aseptically created in the agar plates and 6 mg/ml of the essential oil was added to each well. Streptomycin (20 µg/ml) was added to a well as a positive control to test the bacteria's sensitivity and 10 µl of DMSO was added to the wells as a negative control. The plates were then left at room temperature for a few minutes inside the sterile laminar air flow chamber to allow for proper diffusion of the essential oil from their respective wells into the media. Following this, the plates were incubated at 37 ^{0}C for 24 hours. After the incubation period, the zones of inhibition were measured. This method allows for a qualitative assessment of the antimicrobial activity of the essential oil.

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) was determined following the method outlined by Wayne, 2003. This involved using the broth dilution method to evaluate the individual MICs of the essential oil against selected bacterial strains. The stock solution of essential oil was prepared by dissolving in DMSO in the 1:1 (v/v) ratio. The final volume was then made to 1 ml with MHB. The essential oil was then diluted serially by using MHB. 100 µl of each dilution of the EOs was transferred to microfuge tubes containing 90 µl of the MHB. 10 µl of bacterial suspension were then added to each dilution of the essential oils, and final volume

was made to 200 µl with MHB. Positive control tubes contained only broth and inoculums, but no essential oil, whereas, only DMSO and inoculum were added in the negative control tubes. The tubes were then incubated at 37°C for 24 hours. After incubation, 40µl of p-iodonitrotetrazolium violet solution was added to examine the bacterial growth for another 20 minutes. The tubes were then examined for colour change. Development of pink colour (due to reduction of dye) indicated the bacterial growth. The lowest concentration of essential oil at which no colour change of the dye took place (or no bacterial growth observed) was considered as the MIC for that particular oil.

Statistical analysis: For each measurement, three replicates of each sample were performed subjected to one way analysis of variance (ANOVA) and significant differences between samples were determined using R studio software (Version, 2018).

RESULTS AND DISCUSSION

The antioxidant activity of *Cannabis sativa* essential oil was evaluated using the DPPH radical scavenging assay. The IC_{50} value of bhang essential oil was found to be 71.99 ± 0.34 µg/ml**,** whereas the standard antioxidant **(**BHT**)** exhibited a significantly lower IC_{50} value of 30.05 ± 0.26 µg/ml, indicating its superior free radical scavenging activity (Table 1). The essential oil demonstrated appreciable antioxidant activity, suggesting the presence of naturally occurring bioactive constituents capable of donating hydrogen atoms or electrons to neutralize DPPH free radicals. These findings indicate that essential oil possesses antioxidant potential and may serve as a natural source of antioxidant compounds. The ferric reducing antioxidant power (FRAP) assay was employed to evaluate the reducing ability of *Cannabis sativa* essential oil. The FRAP value of essential oil was recorded as 374.12 ± 0.31 µM Fe^{2+} equivalents/g of dry weight**,** whereas the standard antioxidant **(**BHT) exhibited a significantly higher FRAP value of 1125.84 ± 0.33 µM Fe^{2+} equivalents/g of dry weight, indicating greater reducing power (Table 1). However, the essential oil exhibited measurable ferric ion reducing ability, demonstrating its antioxidant potential.

The antibacterial activity of bhang essential oil was evaluated against five bacterial pathogens *via* agar well diffusion method. The inhibitory activity of the essential oil against the tested bacterial strains is reflected in Table 2. Among the tested microorganisms, the highest antibacterial activity was observed against *Escherichia coli*, with an inhibition zone diameter of 11.70 ± 0.34 mm, followed by *Micrococcus luteus*, showing an inhibition zone of 11.30 ± 0.11 mm. The essential oil also inhibited the growth of *Klebsiella pneumoniae, Staphylococcus aureus* and *Bacillus subtilis*, producing inhibition zone diameters of 10.30 ± 0.26 mm, 10.00 ± 0.23 mm and 8.70 ± 0.26 mm, respectively (Plate 1). Streptomycin (2 µg), used as the positive control, exhibited significantly larger inhibition zones against all bacterial strains, whereas DMSO (99.9%), used as the negative control. These findings indicate that the essential oil possesses moderate antibacterial activity against both gram-positive and gram-negative bacteria, although its efficacy varied among the tested bacterial species. The minimum inhibitory concentration (MIC) of essential oil against the susceptible bacterial strains ranged from 1500 to 5000 µg/ml (Table 3). The lowest MIC value (1500 µg/ml) was recorded against *Escherichia coli*, indicating that this organism was the most susceptible to the essential oil. This was followed by *Micrococcus luteus*, which exhibited an MIC of 2000 µg/ml, while *Klebsiella pneumoniae* showed an intermediate MIC value of 3500 µg/ml. Higher MIC values of 4500 µg/ml and 5000 µg/ml were observed against *Staphylococcus aureus* and *Bacillus subtilis*, respectively, indicating comparatively lower sensitivity. Overall, the MIC values were in agreement with the agar well diffusion assay, where larger inhibition zones corresponded to lower MIC values, confirming that the essential oil possesses moderate antibacterial activity against both gram-positive and gram-negative bacteria.

The data was compared with literature values and it was found that the data was in good agreement with the published data. At a concentration of 500 µg/ml, the essential oil has been reported to exhibit 76.54% DPPH radical scavenging activity and 56.10% FRAP activity, indicating antioxidant capacity. In comparison, the lower DPPH IC_{50} value obtained in the present study reflects a relatively higher free radical scavenging efficiency (Shami *et al*., 2023). The observed difference between the DPPH and FRAP assays suggests that the bioactive compounds present in *Cannabis sativa* essential oil, particularly cannabinoids and terpenes, possess a stronger ability to neutralize stable free radicals via a single-electron transfer mechanism than to reduce ferric (Fe^{3+}) ions into ferrous (Fe^{2+}) ions (Hacke *et al*., 2019). The antioxidant capacity varied among cultivars due to differences in the concentration of major volatile constituents, particularly β-caryophyllene, α-humulene and myrcene (Palmieri *et al*., 2021). Essential oils obtained from different *Cannabis sativa* cultivars have been reported to exhibit antibacterial activity against multi drug-resistant *Staphylococcus pseudintermedius*. The antimicrobial effect was associated with the synergistic interaction between cannabinoids and terpenes, with cultivars containing higher levels of phytocannabinoids demonstrating greater antibacterial efficacy (MIC 23.81 mg/mL; MBC 47.61 mg/mL) than those with lower phytocannabinoid content (MIC 47.61 mg/mL; MBC 95.22 mg/mL) (Pieracci *et al*., 2024). The antibacterial activity is due to hydrophobic constituents of the essential oil, which disrupt

bacterial cell membranes, increase membrane permeability, promote leakage of intracellular contents, and ultimately inhibit microbial growth (Nafis *et al*., 2019).

Table 1. Antioxidant activity of *Cannabis sativa*

Test material	**DPPH Radical Scavenging IC_{50} (µg/ml)**	**FRAP (µM Fe^{2+} equivalents/g of dry wt.)**
Essential oil	71.99 ± 0.34µg/ml (IC_{50})	374.12 ± 0.31
BHT	30.05 ± 0.26	1125.84 ± 0.33

Data is Mean ± S.D. of three replications

Table 2. Antibacterial activity *via* agar well diffusion method of *Cannabis sativa*

Test material	**Conc.**	**Gram positive bacteria**			**Gram negative bacteria**	
		Bacillus subtilis	***Staphylococcus aureus***	***Micrococcus luteus***	***Escherichia coli***	***Klebsiella pneumoniae***
Essential oil	6 mg/ml	8.60 ± 0.24 mm	9.80 ± 0.20 mm	10.20 ± 0.25 mm	**11.50 ± 0.28 mm**	8.90 ± 0.24 mm
Streptomycin (+ve) control	2 µg/ml	20.00± 0.21 mm	20.00± 0.24 mm	18.00± 0.23 mm	18.00± 0.25 mm	17.00 ± 0.26
DMSO-99.9% (-ve) control	10 µl	ND	ND	ND	ND	ND

Data is Mean ± S.D. of three replications
ND: Activity not determined at tested concentration

Table 3. Minimum Inhibitory Concentration (MIC) of *Cannabis sativa*

Essential oil	**MIC (µg/ml)**				
	Bacillus subtilis	***Staphylococcus aureus***	***Micrococcus luteus***	***Escherichia coli***	***Klebsiella pneumoniae***
Essential oil	5000	4500	2000	1500	3500
Streptomycin (+ve) control	2	2	2	2	2

Data is Mean of three replications
ND: Activity not determined at tested concentration

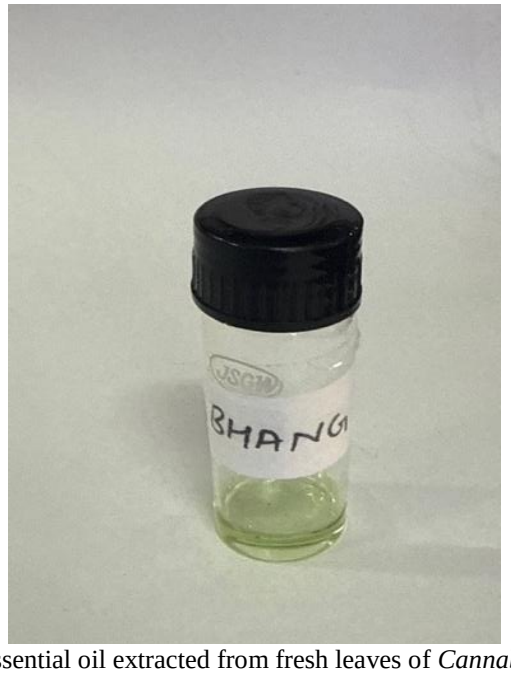

Fig. 1. Essential oil extracted from fresh leaves of *Cannabis sativa*

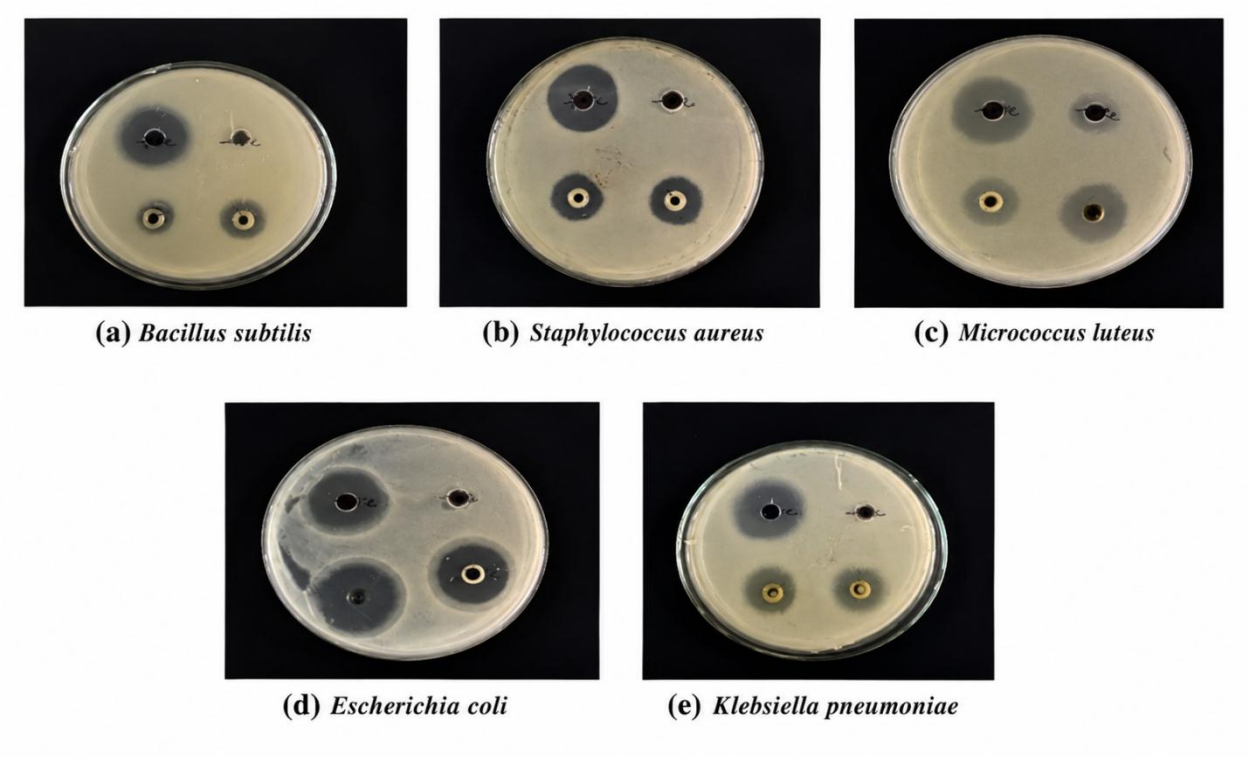

Plate 1. Antibacterial activity of *Cannabis sativa* against a) *Bacillus subtilis* MTCC2389 b) *Staphylococcus aureus* MTCC7443 c) *Micrococcus luteus* MTCC4821 d) *Escherichia coli* MTCC2127 e) *Klebsiella pneumonia* MTCC7172 bacterial strains.

The findings of the present study demonstrated that *Cannabis sativa* essential oil possesses promising antioxidant and broad-spectrum antibacterial activities, highlighting its potential as a valuable natural source of bioactive compounds. The essential oil effectively scavenged free radicals and inhibited the growth of both gram-positive and gram-negative bacterial strains, which may be attributed to the combined action of cannabinoids, terpenes and other phenolic constituents. These compounds are known to neutralize reactive oxygen species, reduce oxidative stress and disrupt bacterial cell membranes, thereby inhibiting microbial growth and proliferation. Owing to these multifunctional biological properties, bhang essential oil shows considerable potential for application as a natural antioxidant and antimicrobial agent in pharmaceutical formulations, food preservation systems and other health-related products as a safer alternative to synthetic additives.

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