Research Article

Cannabis sativa L. (Bhang): A Possible Source of New Antibacterial Medicament

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ABSTRACT

The continuous development of antibiotic resistant pathogens has justified the attempts to search for new therapeutic agents that are able to inhibit the mechanisms that confer resistance to classical drugs. Plant extracts have been used for centuries for treating several ailments and known to contain a wide range of compounds that have antibacterial properties. Taking this into consideration, the ethanol, methanol and aqueous leaf extracts of *Cannabis sativa* were evaluated for their antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* following well diffusion method. Ethanolic extract was found effective against all the tested strains, while aqueous extract showed no inhibitory effect against any of the strains. However, methanolic extract was found ineffective only against *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was also determined for crude ethanolic extract and was found to be 25mg/ml and 50mg/ml respectively. Crude ethanolic extract was further fractioned through column chromatography. Each isolated fractions were again tested for their antibacterial activity. Phytochemical analyses were performed for all the crude extracts as well as for most active ethanolic fractions. Alkaloids, phenol, diterpenes, glycosides, saponins, sterols and flavonoids were found present in the extracts. Our finding suggests that the antibacterial activity of *Cannabis sativa* is due to the antagonistic and synergistic effect of the phytochemicals present in its extracts. Present study confirms the utility of plant for therapeutic purpose and also as an alternate medicine. To further confirm its therapeutic applicability and to strengthen its potential as herbal medicine, there is an urgent need to isolate and characterize its compound followed by its mechanistic, cytotoxic and in-vivo studies.

Keywords: *Cannabis sativa*, antibacterial activity, MIC, MBC, column chromatography, phytochemicals.

INTRODUCTION

Resistance to antibiotics is one of the biggest problem that have become a major clinical and public health concern.[1, 2] It arises mainly due to the adaption of infectious pathogens to antimicrobials used in several areas, including medicine, food, animals, crop production and disinfectants in farms, hospital and households.[3, 4] Bacteria have developed resistance to all commercially available antibiotics and, as so, the economic burden associated with these multidrug-resistant bacteria is high. In order to find novel antibacterial agents with new modes of action, plants have been explored as potent sources for the identification of effective and safe antibacterials.[5] Utilization of plants for therapeutic purposes can be traced back to the beginning of human history. It is estimated that about one quarter of all drugs in modern pharmacopeias are derived from plants.[6, 7] In addition, as in most of the cases, plants or their extracts are believed to be quite safer for humans.[8, 9] According to the World Health Organization (WHO), as much as 80% of the population of developing countries even nowadays depends on plants as their only affordable source of medication.[10] Plants produce a vast array of phytochemicals and it is generally accepted that a significant part of this chemical diversity is related to defence/stress mechanisms including in vitro antibacterial activity.[11] This rich diversity of phytochemicals has partly arisen because of evolutionary selection for enhanced defense mechanisms against a wide array of microorganisms, insects, nematodes and even other plants.[12] In addition to the antibacterial potential, phytochemicals have other beneficial therapeutic effects on mammalian disease prevention/control. For instance, there are a considerable number of reports indicating that phytochemicals can act as antivirals[13], antiparasitics[14], antifungals[15] and can exert a cytotoxic action against tumour cells[16]. Despite the potential therapeutic value of many plant secondary metabolites[17] and the ability of some of them to modify the resistance associated with MDR strains[18], plants are still being less exploited for the development of potential therapeutic products. Considering this, our study has been designed to evaluate the antibacterial activity of *Cannabis sativa* in crude as well as in purified extract with respect to its phytochemical screening.

Cannabis sativa is an angiosperm belonging to the family Cannabaceae.[19] It is known by various names worldwide as Marijuana in America; Bhang, Ganja and Charas in India; Kif in North Africa; Dogga in South Africa; Krori in Tunisia, Habak in Turkey; Hashish in Middle East; Djomba or Liamba in Central Africa and Brazil; Sodom, Tampl, Gum, Gauge and stuff in Kinshasa, Swala and Whiskt in Ghana; Grifa in Mexico and Macohna in some parts of South America.[20] The plant grows well at low temperature, and well-adjusted to moderate climates.

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Cannabis sativa extracts as medicine was described in China and India before the birth of Christ.[21] The plant and its preparations have been used for its sedative, narcotic, antispasmodic, analgesic, antimicrobial and many other properties including its use for photophobia, asthma and piles.[22, 23] *Cannabis* also induces an increase in heart rate, lowers blood pressure due to vasodilatation and stimulates appetite.[24] Its extracts may represent an efficacious and safe alternative for treating insomnia, sick headaches, neuralgia, migraine, mania, whooping cough, asthma, dysuria and in relieving pain in dysmenorrhoea and menorrhagia.[25-27] *Cannabis* is being used as a shampoo and for other cosmetic purposes.[28] It is administered to patients suffering from rabies, cholera, rheumatism, epilepsy and tetanus. Also observation is that, *Cannabis sativa* have been used for the treatment of specific human ailments such as allergies, burns, cuts and wounds, inflammation, leprosy, leucoderma, scabies, smallpox and sexually transmitted diseases.[29] These observations would suggest that, *Cannabis sativa* can be exploited to develop effective and safe therapeutic agents with new modes of action, so as to combat resistant pathogens.

MATERIALS AND METHODS

Sample Collection and Preparation of Plant Extracts

Cannabis sativa was collected in October, 2014 (moderate climate in northern India), from nearby area of Amity University Uttar Pradesh, Lucknow Campus. The plant leaves were washed thoroughly with tap water followed by distilled water to remove the dust particles and allowed to air dry at room temperature on laboratory bench. The dried plant leaves were pulverized with the help of liquid nitrogen and stored at -20°C till further use. For preparing extract, 10 gm of the pulverized sample was mixed with 100 ml methanol, ethanol and distilled water. The mixture was macerated in mortar and pestle and kept for 48 hrs at room temperature to ensure maximum metabolite extraction. The extract obtained was filtered and concentrated. The final concentration was maintained as 100mg/ml by redissolving the crude extracts in 10% dimethylsulfoxide[30] for bioassay analysis.

Test Organisms and Bacterial Inoculum Preparation

The bacteria used in this study included *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. These strains were obtained from HiMedia Laboratory Pvt. Ltd. and maintained by sub culturing on blood agar base No. 2 (HiMedia Laboratory Pvt. Ltd.) and macconkey's agar (HiMedia Laboratory Pvt. Ltd.) plates prior to use. To prepare bacterial inoculum, pure isolates of bacteria were diluted in test tubes containing 0.9% normal saline solution followed by 15min incubation in an ambient air incubator (Thermo Scientific, Thermo electron LED GmbH) so as to meet the 0.5 McFarland turbidity standard (TULIP DIAGNOSTICS (P) LTD.), which is equivalent to 1.5 x 10^8CFU/ml before applying onto the plates. For MIC testing, standardized inoculum should have a desired concentration of 5x10^5CFU/ml which is prepared by delivering 1ml of the adjusted suspension (1.5 x 10^8CFU/ml) in 25ml of water.[31]

Antibacterial Activity Assay

The agar gel diffusion assay[33] was carried out on Muller Hinton Agar No. 2 (HiMedia Laboratory Pvt. Ltd.) plates to assess antibacterial activity of the extracts. The media was prepared as per the supplier's instructions and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min (High-pressure steam sterilizer ES-315, TOMY KOGYO CO., LTD). The plates were then inoculated with the diluted bacterial suspensions using sterile swab sticks dipped in it. Wells of 6 mm size were dug with the help of a sterile cork borer on these plates. In each wells, 100 µl of the methanol, ethanol and aqueous extracts were loaded taking care not to allow spillage of the solutions onto the surface of the agar. The culture plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of these extracts before being incubated at 37^0c for 24 h. After incubation period, the antibacterial activity was calculated in terms of zone of inhibition (diameter in mm). Determinations were done in triplicates and mean of all values was taken. Standard antibiotic (Gentamicin) was used as positive control, while DMSO (10%) was used as negative control to compare the antibacterial activity of the extracts.

Phytochemical Screening

Phytochemical examinations of alkaloids, carbohydrates, glycosides, saponins, sterols, phenols, tannins, flavonoids, proteins, amino acids, and diterpenes were carried out for all the crude as well as most active purified extracts as per the standard methods.[32]

Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration which results in the reduction of inoculums viability.[34] The MIC of the crude extract (ethanolic) of *Cannabis sativa* was determined by broth macro dilution method.[31, 35] The growth media, nutrient broth (HiMedia Laboratory Pvt. Ltd.) was prepared as per the supplier's instructions and sterilized by autoclaving. The sterilized media was allowed to cool to 50˚C and 2ml was added to each labeled test tubes as per concentrations taken. Serial dilution of the plant extract (100mg/ml) was done by transferring 2ml from each test tube to obtain two fold dilutions (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml 1.56mg/ml). The mixture of the media and the test crude extract were thoroughly mixed and 100µl of the test organism (*Staphylococcus aureus*) having desired inoculum concentration of 5x10^5 was added to all the test tubes. The tubes were then incubated at 37^OC for 24 h and MIC were expressed as the highest dilution which inhibited growth, determined by lack of opacity in the tubes. Two blank nutrient broth tubes with bacterial inoculation were used as the growth controls, of which one is kept overnight at 4°C in a

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refrigerator for determination of complete inhibition, as very faint turbidity may be given by the inoculums itself. Also a nutrient broth tube without bacterial inoculation was used as the sterility control. 10% DMSO was used as negative control while broth containing standard drug (Gentamicin) was used as positive control.

Minimum Bactericidal Concentration (MBC)

The MBC is the lowest concentration of an antibacterial agent required to kill a particular bacteria.[36] It was determined by sub culturing from each tubes of MIC showing no apparent growth. Before being subcultured, the tubes were gently mixed by flushing them with a sterile pipette. Each aliquot was then spread over the fresh Muller-Hinton Agar plates by lawning technique. The MBC lawned plates were incubated at 37°C for 24 h. After the incubation period, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for this extract.[37] This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

Fractionation of Extract through Column Chromatography

Crude ethanolic extract of *Cannabis sativa* was further fractioned through column chromatography using a single solvent throughout the process. Column was filled with silica gel (60-120 mesh) which acts as a stationary phase and packed by passing eluant (ethanol) acting as a mobile phase. After sample was added (ratio stationary phase to crude extract 5:1), each fraction was collected by passing eluant from top of the column until silica gel appears colorless. Antibacterial activity assay was performed for each fraction through the same procedure as for crude extract.

RESULTS AND DISCUSSION

The antibacterial activity of methanol, ethanol and aqueous leaf extracts of *Cannabis sativa* were observed using agar well diffusion method by measuring the diameter of the zone of inhibition (Table 1). Out of three extracts of the plant, ethanolic extract was found effective against all the tested strains and showed moderate (19mm) activity against *Staphylococcus aureus*, while mild activity against *Pseudomonas aeruginosa* (13mm) and *Escherichia coli* (12mm). Methanol extract of *Cannabis sativa* showed moderate and mild antibacterial activity against *Staphylococcus aureus* (17mm) and *Escherichia coli* (11mm) but found ineffective against *Pseudomonas aeruginosa.* However, aqueous extract showed no inhibitory effect against all the tested strains. All the isolates were susceptible to standard antibiotic (Gentamicin) tested with appreciable zone of inhibition measured as- for *Pseudomonas aeruginosa*-28mm, *Staphylococcus aureus*- 30mm and *Escherichia coli*-28mm, but the negative control (DMSO) was found ineffective against all the tested strains. The Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between the two and/or in their genetic contents.[38] The cell walls of Gram-negative organisms are more complex in lay out than the Gram-positive ones acting as a diffusion barrier and making them less susceptible to the antimicrobial agents than Gram-positive bacteria.[39, 40, 41]

In the present study *Cannabis sativa* was also subjected to various biochemical tests (Table 2) in order to determine the active constituents present in its crude as well as most active fractions of the extracts. Ethanolic extract of *Cannabis sativa* was found to contain significant amount of alkaloids, glycosides, phenol and diterpenes, which is represented by +++ sign in the table. In the methanol extract alkaloids was found to have significant (+++) degree of presence, while phenol and diterpene were found with a moderate (++) degree of presence. Furthermore, Glycosides, flavonoids and diterpenes were found in a significant (+++) amount in the aqueous extract of the plant, while alkaloids and saponins were found with a moderate (++) and sterols with a mild (+) degree of presence. After fractionation, the most active fractions (F1, F2, F3 and F5) were again subjected for phytochemical examination. The activity of the different fractions was determined by the same method as for crude extracts. Fraction 1 and 2 were found to contain significant (+++) and moderate (++) amount of diterpenes. Significant (+++) and moderate (++) amount of glycosides was found in fraction 2 and 3, while alkaloids and phenol were found with a moderate (++) degree of presence in fraction 5.This finding would suggest that, antibacterial activity of the plant is due to the presence of these components.

The various phytochemicals detected are known to have beneficial importance in medicinal sciences.[32] Alkaloids are heterocyclic nitrogen compounds, responsible for plant defense against enteric pathogenic organisms and are widely used as pharmaceuticals, psycho-stimulants, narcotics, and poisons due to their renowned biological activities.[42] They have been found to have microbiocidal effects[43] with their ability to intercalate with DNA.[44] Phenols are chemical components that occur universally as natural color pigments responsible for the color of fruits of plants. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL).[42] The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with the fact that increased hydroxylation results in increased toxicity.[45] In addition, some authors have found that more highly oxidized phenols are more toxic[46, 47]. The mechanisms of action responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins.[48]

The fragrance of plants is due to the presence of essential oil fraction. These oils are secondary metabolites that are

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highly enriched in compounds based on anisoprene structure and are called terpenes. Theirgeneral chemical structure is C10H16, and occurs as diterpenes, triterpenes, tetraterpenes, hemiterpenes and sesquiterpenes (C20, C30, C40, $C_{5,}$ and C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids.[49] Terpenes or terpenoids are active against bacteria[50, 51], fungi[52, 53], viruses[54, 55] and protozoa.[43, 56] The mechanism of action of terpenes is not fully understood but is thought to involve membrane disruption by the lipophillic compounds. Flavonoids are important group of polyphenols widely distributed among the plant flora. Their structure contains one carbonyl group (as opposed to the two carbonyls in quinones), which occur as a C6-C3 unit linked to an aromatic ring.[49] Since they are known to be synthesized by plants in response to microbial infection[57] it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide range of microorganisms. Their activity is probably due to their ability to form complexes with extracellular and soluble proteins as well as with bacterial cell walls, thereby inducing microbial cell membrane disruption.[58] Saponins are compounds with 'soaplike' behavior in water. They exert antibacterial activity by combining with cell membranes to elicit changes in cell morphology leading to cell death.[58] They are also used in hypercholesterolemia, hyperglycemia and also have antioxidant, anticancer, anti-inflammatory and antifungal activities.[59] Glycosides are the condensation products of sugars with different varieties of organic hydroxyl or thiol compounds.[42] It serves as plant defense against predation by many microorganisms, insects and herbivores.[60]

Minimum inhibitory concentration (MIC) of the crude ethanolic extract was also detected following broth macro dilution method (Table 3). Ethanolic extract was selected due to its inhibitory activity against all the tested strains. The test was done against *Staphylococcus aureus*, because of its greater activity amongst others. The tubes of MIC were further subcultured on fresh Muller-Hinton agar plates, in order to determine minimum bactericidal concentration (MBC) of the extract (Table 4). The MIC and MBC values of the extract was found to be 25mg/ml and 50mg/ml. MBC assay result confirms the data of agar well diffusion assay (Table 1) and the MIC determination assay (Table 3). These results further confirm the utility of ethanolic leaf extract of *Cannabis sativa* in developing potential and safe antibacterials with its minimum concentration.

Crude ethanolic extract of the plant was further subjected for fractionation. From column chromatography six fractions were collected. Different fractions showed different antibacterial activity (Table 5) with the zone of inhibition measured as- fraction 1 (22, 10, 13mm), fraction 2 (26, 21, 12mm), fraction 3 (R, 20, 11mm), fraction 4 (12, 10, 11mm), fraction 5 (25, 11, 11mm) and fraction 6 (12, 11, 10mm) against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*. The extracts (crude and purified) were found to be less potent than the standard antibiotic. The differences between the activities of the extracts and the standard drug may be due to the mixture of compounds present in the extracts compared to the pure compound contained in the standard antibiotics.[61] It has also been observed through the study that, ethanolic and methanolic extracts were effective against both gram-positive and gram-negative bacteria, which indicates the potency of these extracts to be used in the development of broad spectrum antibiotics and may be helpful in eradicating bacterial resistance problems worldwide. The results of the present study also indicate that, crude ethanolic extract showed greater antibacterial activity against *Staphylococcus aureus* compare to other tested strains, whereas *Pseudomonas aeruginosa* was found inhibited with greater zone of inhibition by some purified fractions of the ethanolic extract than the other tested strains. The differences in the antibacterial activity of the crude and purified extracts were may be due to antagonistic and synergistic effect of the phytochemicals present in it. The combinations of the various phytochemicals present in the crude extract may act synergistically against *Staphylococcus aureus* and antagonistically against *Pseudomonas aeruginosa*, while after fractionation the components of the extract got separated and act synergistically towards *Pseudomonas aeruginosa*, making it more susceptible than the other tested strains. However, phytochemicals present in the crude aqueous extract may act antagonistically, making it completely resistant against all the tested strains.

Table 1: Zone of inhibition of crude extracts of *Cannabis sativa*

Test Organisms	Zone of Inhibition (mm)				
	Standard Antibiotic	Extracts (100mg/ml)			DMSO (10%)
	Gentamicin (85mg)	Methanol	Ethanol	Aqueous	
Pseudomonas aeruginosa	28	R	13	R	R
Staphylococcus aureus	30	17	19	R	R
Escherichia coli	28	11	12	R	R

Avg. ZOI:< 15mm (mild), <25mm (moderate), >25mm (significant); R: Resistant
Values are average mean of the triplicate samples

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Table 2: Phytochemical screening of crude extracts and most active ethanolic fractions of *Cannabis sativa*

S. No.	Test Name	Crude extracts			Ethanolic fractions			
		Ethanol	Methanol	Aqueous	F1	F2	F3	F5
1	**Alkaloids**							
	Wagner's test	+++	+++	++	-	-	-	++
2	**Carbohydrates**							
	Benedict's test	-	-	-	-	-	-	-
3	**Glycosides**							
	Mod. Borntrager's test	+++	-	+++	-	+++	++	-
4	**Saponins**							
	Froth test	-	-	++	-	-	-	-
5	**Sterols**							
	Salkowski's test	-	-	+	-	-	-	-
6	**Phenol**							
	Ferric chloride test	+++	++	-	-	-	-	++
7	**Tannins**							
	Gelatin test	-	-	-	-	-	-	-
8	**Flavonoids**							
	Alk. Reagent test	-	-	+++	-	-	-	-
9	**Proteins**							
	Xanthoproteic test	-	-	++	-	-	-	-
10	**Amino acids**							
	Ninhydrin test	-	-	-	-	-	-	-
11	**Diterpenes**							
	Copper acetate test	+++	++	+++	+++	++	-	-

(+++): Significant degree of presence; **(++):** Moderate degree of presence; **(+):** Mild degree of presence; **(-):** No presence

Table 3: MIC of the crude ethanolic extract of *Cannabis sativa* against *Staphylococcus aureus*

Test organism	Concentration (mg/ml)						
	100	50	25	12.5	6.25	3.125	1.56
Staphylococcus aureus	+	+	+	_	_	_	_

(+): Clear broth, indicating no growth; **(-):** Turbidity in the broth, indicating growth

Table 4: MBC of the crude ethanolic extract of *Cannabis sativa* against *Staphylococcus aureus*

Test organism	Concentration (mg/ml)		
	100	50	25
Staphylococcus aureus	+	+	-

(+): No colonies on petriplates observed; **(-):** Petriplate observed with bacterial colonies

Table 5: Zone of inhibition of ethanolic fractions of *Cannabis sativa*

Test organisms	ZOI of ethanolic fractions					
	1	2	3	4	5	6
Pseudomonas aeruginosa	22	26	R	12	25	12
Staphylococcus aureus	10	21	20	10	11	11
Escherichia coli	13	12	11	11	11	10

Avg. ZOI: < 15mm (mild), <25mm (moderate), >25mm (significant); R: Resistant
Values are average mean of the triplicate samples

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CONCLUSION

The antibacterial activity of the different extracts of *Cannabis sativa* depends on the various phytochemicals present in it. The phytochemicals in the extracts exerts their effects by acting synergistically and antagonistically towards different strains. It is clear from the study that, crude ethanolic leaf extract of plant may be considered as the good choice for developing new effective antibacterials to treat diseases caused by *Staphylococcus aureus*, whereas to treat infections/diseases caused due to *Pseudomonas aeruginosa*, purified ethanolic extract came out to be the good choice. However, *Escherichia coli* was found to be highly resistant (though not completely resistant) in both crude and purified ethanolic extracts, indicating less efficacy of the plant against this strain. Present study also indicate that, ethanolic extract of *Cannabis sativa* can be included in the list of herbal medicines in appropriate concentrations due to its bactericidal effect and hence can be recommended for therapeutic purpose and can be used as an alternate medicine. To further confirm its therapeutic applicability and to strengthen its potential as herbal medicine, there is an urgent need to isolate and characterize its compound followed by its mechanistic, cytotoxic and in-vivo studies.

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