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Antimicrobial Potential of Three Common Weeds of Kurukshetra: An *in vitro* Study

[1]Sahaj Kaur, [2]Chetan Sharma, [1]Smita Chaudhry and [3]Robina Aman
[1]Institute of Environmental Studies, Kurukshetra University, Kurukshetra, 136119, Haryana, India
[2]Department of Biotechnology, Maharishi Markandeshwar University, Mullana, Ambala, 133207, India
[3]Department of Chemistry, Kumaun University, S.S.J. Campus Almora, 263601, India

Corresponding Author: Chetan Sharma, Department of Biotechnology, Maharishi Markandeshwar University, Mullana, Ambala, 133207, India

ABSTRACT

In the present study a comparison on antimicrobial potency of three common weeds i.e. *Cannabis sativa*, *Parthenium hysterophorus* and *Calotropis procera*, were tested by agar well diffusion method against the six common pathogens namely, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Saccharomyces cerevisiae*. The *in vitro* study revealed that organic leaves extract of weeds were more effective than aqueous extract. Among the three tested weeds, methanolic extract of *C. sativa* was found to be most effective as compare to other weeds. It showed maximum zone of inhibition of 26.3 and 25.6 mm against *Bacillus subtilis* and *Staphylococcus aureus* with lowest MIC of 1.56 mg mL^{-1}. This study revealed that the organic leaves extract of *C. sativa* showed good antibacterial activity and can be explored for developing antibacterial drugs.

Key words: Weeds, antimicrobial activity, minimum inhibitory concentration

INTRODUCTION

Over the last years the incidence of infectious diseases and the development of antibiotic resistant pathogens in developing countries justify the attempts to search for new antimicrobial agents as well as for compounds that are able to inhibit the mechanisms that confer resistance to classical drugs. Plants extracts have been used for centuries for treating several ailments and known to contain a wide range of compounds (secondary metabolites) that have antimicrobial properties and there are many taxa that have not been evaluated (Sharma *et al*., 2011; Madureira *et al*., 2012; Prakash *et al*., 2012). A weed is commonly defined as a plant that grows out of place and is competitive, persistent and pernicious. Weeds have been a part of civilization and many ancient documents speak of humans battling weeds in the crops they grow (James and Evans, 1991). If weed contains antimicrobial properties, it will act as boon to mankind.

Cannabis sativa is a member of the family *Cannabinaceae*, commonly known by various names worldwide as Marijuana in America; Bhang, Ganja and Charas in India and Hashish in Middle East (Sachindra and Pradhan, 1977). *Cannabis* is administered to patients suffering from rabies, cholera, rheumatism, epilepsy and tetanus. In Northeastern India, it has 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. Cannabinoids, a major secondary metabolite found in the cannabis plant, responsible for the plant peculiar

pharmacological effects (Lambert and Fowler, 2005). *Parthenium hysterophorus* L. (Asteraceae), is an invasive weed throughout world. In India it was first reported in the late 1950s and commonly known as altamisa, carrot grass, bitter weed, star weed, white top, wild feverfew, the Scourge of India and the congress grass. Plant has been used as folk remedy for the treatment of infectious and degenerative diseases. All parts of the plant are reported to be used as bitter tonic, febrifuge, emmenagogue, antidysenteric etc. Some researchers have reported its use in traditional medicine for treatment of wounds, ulcerated sores, fever, anemia and heart troubles. The leaf extracts have role in the fertility, fecundity and behavioral response. Parthenin, a sesquiterpenoid is the major active compound present in this plant (Kumar *et al*., 2013a). *Calotropis procera* Linn. (Asclepiadaceae) also known as milkweed is a xerophytic, erect shrub about 6 m high, growing widely throughout the tropic of Africa and Asia. It is known as Aak, Sodom apple and Usher. It has been reported for its wound healing, analgesic, acaricidal, antinociceptive, antiulcer and anti-coccidial activity. *Calotropis* has shown the presence of triterpenoids, calotropursenyl acetate, calopfriedelenyl, procerleanol A and B, cardiac glycosides, calotropin, calotoxin, cardenolides and anthocyanins (Meena *et al*., 2011; Sharma *et al*., 2012; Kumar *et al*., 2013b).

Literature search reveals conducting of a very few studies on antimicrobial activity of weeds (Bhuvaneswari *et al*., 2011; Manikandan *et al*., 2011; Prakash *et al*., 2012; Sanguri *et al*., 2012). The present investigation is therefore, undertaken to test the efficacy of some common weeds extracts against the bacterial and fungal pathogens.

MATERIALS AND METHODS

Plant collection and its extraction: The leaves of three weeds namely *Cannabis sativa, Parthenium hysterophorus* and *Calotropis procera* were collected from the barren lands of Kurukshetra, Haryana. The taxonomic identities of these weeds were confirmed by Dr. BD Vashishta, plant taxonomist, Botany Department, Kurukshetra University, Kurukshetra. The collected leaves were inspected for their pathogenic infections and healthy leaves were selected after examining it carefully. The leaves were carefully washed under running tap water followed by sterile distilled water and air dried at a temperature of 38°C for 4-5 days, then homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. Four different solvents, namely ethanol, methanol, acetone and aqueous, were used for extraction. Homogenized leaves, 10 g each, were separately soaked in conical flasks each containing 100 mL of acetone, ethanol, methanol (95%) and sterile distilled water. Keep all the flasks on rotary shaker at 200 rpm for 24 h. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rota evaporator. The dried extract thus obtained was sterilized by overnight ultra violet-irradiation, checked for sterility on nutrient agar plates and stored at 4°C in labelled sterile bottles until further use (Aneja and Sharma, 2010; Aneja *et al*., 2011).

Source of microorganisms: A total of six microbial cultures were selected on the basis of their clinical importance in causing diseases in humans. Of the six microbial cultures, 4 bacterial and 2 fungal cultures were used in this study. Two Gram-positive bacteria (*Staphylococcus aureus* MTCC 96 and *Bacillus subtilis* MTCC 121), two Gram-negative bacteria (*Escherichia coli* MTCC 1652 and *Pseudomonas aeruginosa* MTCC 741) and two yeast, *Candida albicans* (MTCC 227) and *Saccharomyces cerevisiae* (MTCC 170) were used in the present study for evaluation of antimicrobial activity of weeds. All the microbial cultures were procured from Microbial Type

Culture Collection (MTCC), IMTECH, Chandigarh. The bacteria were subcultured on Nutrient agar, whereas, yeast were grown on Sabouraud's dextrose agar at 37°C for 24-48 h. The media used in this study were procured from Hi Media Laboratory Pvt. Ltd., Bombay, India.

Antimicrobial assay: The acetone, methanol, ethanol and aqueous leaves extracts of weeds were used for evaluation of antimicrobial activity by agar well diffusion method. In this method, a pure isolate of each microbe was grown on agar plates at 37°C for 24 h. One plate of each microorganism was taken and a minimum of four colonies were transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted to that of 10^6 CFU mL^{-1} (standardized by 0.5 McFarland standard) and used as the inoculum for performing an agar well diffusion assay. One hundred microliter (100 µL) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8 mm wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with molten agar medium. The dried extracts were reconstituted to 20% in dimethylsulphoxide (DMSO) for the bioassay analysis. A 100 µL volume of each extract was propelled directly into the wells (in triplicate) of the inoculated agar plates for each test organism. The plates were allowed to stand for 1 h at room temperature (40°C) for diffusion of the extract into agar and incubated at 37°C for 24 h. DMSO served as the negative control whereas Ciprofloxacin was used as positive control for bacteria and amphotericin-B for fungi. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone was greater than 8 mm. The experiments were performed in triplicate and the mean values of the diameter of inhibition zones±standard deviations were calculated (Aneja *et al*., 2011).

Determination of minimum inhibitory concentration: The MIC is defined as the lowest concentration of a compound/extract that completely inhibits the growth of the microorganism or at which there is no visible growth of the microorganism. The MIC of various crude extracts for each tested bacterium and fungi was determined by the modified agar well diffusion method (Aneja *et al*., 2011). A twofold serial dilution of each extract was prepared by first reconstituting the dried extracted plant material (100 mg mL^{-1}) in DMSO followed by dilution in sterile distilled water to achieve a decreasing concentration range of 50-0.39 mg mL^{-1}. A 100 µL volume of each dilution was introduced into triplicate wells of the agar plates already seeded with 100 µL of standardized inoculum (10^6 CFU mL^{-1}) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24 h and observed for inhibition zones. The MIC, taken as the lowest concentration of the extract that completely inhibited the growth of the microbe, showed by a clear zone of inhibition (>12 mm), was recorded for each test organism (Nostro *et al*., 2000; Adeniyi and Ayepola, 2008).

Statistical analysis: The experimental results were repeated thrice in triplicate each time and expressed as Mean±SD and results were statistically evaluated using Dennett's T-test. p- value less than 0.01 was considered significant.

RESULTS

The antibacterial activity of weeds extracts on the agar plates varied in different organic (methanol, ethanol and acetone) and aqueous extracts. Positive controls produced significantly sized inhibition zones against the tested bacteria (ranging between 27.6 and 22.0 mm) and the

Table 1: Antimicrobial activity of three different weed extracts

Solvent extracts (mg mL^{-1})	Diameter of inhibition zones (mm)					
	Sa	Bs	Pa	Ec	Sc	Ca
Cannabis sativa						
Methanol	25.6[1]±0.57[2]	26.3±0.57	-	16.3±0.57	-	-
Ethanol	21.0±0	22.6±0.57	-	14.0±0	-	-
Acetone	22.6±0.57	24.6±0.57	-	14.3±0.57	-	-
Aqueous	16.6±0.57	19.3±0.57	-	12.6±0.57	-	-
Parthenium hysterophorus						
Methanol	23.6±0.57	25.3±0.57	-	13.6±0.57	-	-
Ethanol	20.6±0.57	21.6±0.57	-	12.3±0.57	-	-
Acetone	21.3±0.57	22.3±0.57	-	13.0±0	-	-
Aqueous	15.6±0.57	17.3±0.57	-	-	-	-
Calotropis procera						
Methanol	17.3±0.57	18.6±0.57	-	-	-	-
Ethanol	13.6±0.57	15.0±0	-	-	-	-
Acetone	14.6±0.57	16.3±0.57	-	-	-	-
Aqueous	-	-	-	-	-	-
DMSO	-	-	-	-	-	-
Ciprofloxacin	27.6±0.57	26.0±0	25.0±0	22.0±0	nt	nt
Amphotericin B	nt	nt	nt	nt	19.3±0.57	16.6±0.57

[1]Values, including diameter of the well (8 mm), are means of three replicates, [2]±Standard Deviation, The data was analyzed by one way analysis of variance followed by Dunnett's test. ap<0.01 vs positive control, Sa: *Staphylococcus aureus*, Bs: *Bacillus subtilis*, Pa: *Pseudomonas aeruginosa,* Ec: *Escherichia coli*, Sc: *Saccharomyces cerevisiae,* Ca: *Candida albicans,* nt: Not tested, -: No activity

yeast (with zone of inhibition ranging between 19.3 and 16.6 mm) and the negative control produced no observable inhibitory effect against any of the test organism (Table 1). A perusal of the data in Table 1 reveals that all the tested solvent extracts possessed antibacterial activity against the tested bacterial pathogens except *Pseudomonas aeruginosa*. The zones of inhibition recorded by different weeds using different solvent against the bacteria studied are presented in Table 1. However, all the three tested weed extracts did not exhibit any antifungal activity. Among the three organic extract used, methanolic extract has been found most effective showing the maximum zone of inhibition as compared to the ethanolic and acetonic extracts and lowest MIC against the tested bacterial pathogens.

Of all the tested extracts from *Cannabis sativa* leaves, methanolic extract was found to be most active. It showed marked antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* with zone of inhibition of 26.3 and 25.6 mm followed by *Escherichia coli* (16.3 mm). The ethanolic, acetonic and aqueous leaves extracts showed moderate activity with inhibition zones of 24.6 and 12.6 mm. *B. subtilis* was found most sensitive pathogen having an MIC of 1.56 mg mL^{-1}. Other tested pathogens were found to be less sensitive, having MIC values between 6.25 and 50 mg mL^{-1}. In case of *Parthenium hysterophorus*, methanolic leaves extract was again found to be most active as compared to the other tested extracts, with maximum zone of inhibition of 25.3 mm (*B. subtilis*) followed by 23.6 mm (*S. aureus*) and 13.6 mm (*E. coli*). However, aqueous extract showed maximum zone of inhibition of 17.3 mm. *B. subtilis* was found most sensitive pathogen having an MIC of 1.56 mg mL^{-1} and *E. coli* was found to be least sensitive pathogen having an MIC of 50 mg mL^{-1} (Table 1-2).

Of the three tested weeds, *Calotropis procera* leaves extract was found to be less active as compared to above described weeds. It shows activity against Gram Positive bacteria only, with maximum zone of inhibition of 18.6 mm (*B. subtilis*) and 17.3 mm (*S. aureus*) and lowest MIC of 25 mg mL^{-1}. Aqueous extract donot exhibit any antimicrobial activity. In the present study, the standard antibiotic, Ciprofloxacin and amphotericin B consistently displayed superior potency

Table 2: Minimum inhibitory concentration of three different weeds extracts

	MIC (mg mL^{-1})			
Solvent extracts (mg mL^{-1})	Sa	Bs	Pa	Ec
Cannabis sativa				
Methanol	1.56	1.56	nt	50
Ethanol	6.25	6.25	nt	50
Acetone	6.25	3.12	nt	50
Aqueous	25	12.5	nt	>50
Parthenium hysterophorus				
Methanol	3.12	1.56	nt	50
Ethanol	6.25	6.25	nt	>50
Acetone	6.25	6.25	nt	50
Aqueous	50	25	nt	nt
Calotropis procera				
Methanol	25	25	nt	nt
Ethanol	50	50	nt	nt
Acetone	50	50	nt	nt
Aqueous	nt	nt	nt	nt

Sa: *Staphylococcus aureus*, Bs: *Bacillus subtilis*, Pa: *Pseudomonas aeruginosa*, Ec: *Escherichia coli* and nt: Not tested, MIC: Maximum inhibitory concentration

when compared with the tested crude extracts. However, methanolic extract of *C. sativa* showed antibacterial activity almost equal to the positive control against Gram positive bacteria. All the obtained results were statistically significant as they showed ($p<0.01$) compared with control (Table 1).

DISCUSSION

Emergence of multi-drug resistance in human pathogenic microorganims as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin. Man has been using plants either in pure forms or crude extracts, since time immemorial. Bioactive compounds from these plant sources have been isolated and characterized worldwide and systematic screening of plant materials represent an important effort to find some new bioactive compounds with the high therapeutic potential to fight against pathogenic microorganisms. The elucidation of the chemical structures of some of these compounds had led to the synthesis and production of more potent and safer drugs (Parekh and Chanda, 2007; Sharma *et al*., 2011). The potency of weed plants to grow at extreme conditions can be exploited properly for mankind. The present study reveals the importance of weed plants in controlling bacterial infections. The antibacterial and antifungal properties of the leaf extracts of three different weeds on the test isolates were revealed in Table 1.

All the tested weeds extracts showed varied amount of antibacterial activity this might be due to the presence of phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic compounds that act as secondary metabolites of plants. This can partially explain the demonstration of antibacterial activity by the leaves extracts of weeds (Bonjar *et al*., 2004). Of the three tested weeds, *C. sativa* showed better antibacterial activity as compare to *P. hysterophorus* and *C. procera*. *C. sativa* possess a variety of distinct chemical classes. The classical cannabinoids are structurally related to Tetrahydrocannabinol cannabinoids, a unique group of secondary metabolites found in the plant responsible for pharmacological effects. Currently, there are three general types of cannabinoids; phytocannabinoids occur uniquely in the cannabis plant; endogenous cannabinoids are produced in the bodies of humans and animals and synthetic cannabinoids which are similar compounds produced in the laboratory (Lambert and Fowler, 2005; Ali *et al*., 2012).

Out of the four solvents used for extraction in our study, methanolic extracts showed the highest activity against the tested organisms, followed by acetonic, ethanolic and aqueous extracts. Different solvents have been reported to have the capacity to extract different phytocompounds depending on their solubility or polarity in the solvent. The traditional practitioners use water as a primary solvent but according to our results depicted in Table 1 showed that methanol was a better solvent for extracting antimicrobial substance from these weeds compared to water. This may be due to better solubility of the active compounds in organic solvents (De Boer *et al*., 2005; Salama and Marraiki, 2010).

Alcoholic extracts of tested plants provided better antibacterial effect than other solvents. The effectiveness of the extracts largely depends on the type of solvent used, where the organic extracts provided more powerful antimicrobial activity as compared to aqueous extracts (Sen and Batra, 2012). Cowan (1999) mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents. Seyydnejad *et al.* (2010) also studied the effect of different alcoholic viz. ethanol and methanol for antimicrobial activity and observed that this difference in the activity between different alcoholic extract is due to the difference between extract compounds in this two extract. Our results are in accordance with other studies, which showed methanol as a better solvent (Bhuvaneswari *et al*., 2011; Prakash *et al*., 2012).

From the results of antibacterial activity of the plant extracts against the bacteria it has been observed that plant extracts are resistant to *Pseudomonas aeruginosa.* The lack of susceptibility of *P. aeruginosa* to the plant extract could be attributed to the fact that this bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane. Also its tendency to colonies in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics.

In our study, the antibacterial activity of plant extract appears to be more inhibitory to Gram-positive bacteria than Gram-negative bacteria. Unlike Gram-positive bacteria, the lipopolysaccharide layer along with proteins and phospholipids are the major components in the outer surface of Gram-negative bacteria. The outer lipopolysaccharide layer hinders access of most compounds to the peptidoglycan layer of the cell wall. This explains the resistance of Gram-negative strains to the lytic action of most extracts exhibiting activity. The negative results obtained against Gram-negative bacteria were not unexpected since this class of bacteria is usually more resistant than Gram-positive bacteria (Kirtikar and Basu, 1968; Sheu and Freese, 1973; Turnbull and Kramer, 1991).

CONCLUSION

From this study it is concluded that among the three common weeds studied, *C. sativa* showed better antibacterial activity than *P. hysterophorus* and *C. procera.* This study suggests that such weeds can be exploited to prepare potent antibacterial drugs and such study will push other researchers to explore the pharmacological activity of unexplored weeds inhabited in and around India.

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