Determination of the Antimicrobial Effects of Hydro-Alcoholic Extract of *Cannabis Sativa* on Multiple Drug Resistant Bacteria Isolated from Nosocomial Infections

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Received: 31.04.2013 *Accepted: 26.05.2013*

ABSTRACT

Background: The science of identification and employment of medicinal plants dates back to the early days of man on earth. Cannabis (hashish) is the most common illegal substance used in the United States and was subjected to extensive research as a powerful local disinfecting agent for mouth cavity and skin and an anti-tubercular agent in 1950.

Methods: Clinical strains were isolated from hospitalized patients in Vali-e-Asr Hospital of Arak. The hydro-alcoholic extract of cannabis (5 g) was prepared following liquid-liquid method and drying in 45˚C. The antimicrobial properties of the extract were determined through disk diffusion and determination of MIC (Minimum Inhibitory Concentration).

Results: First, the sensitivity of bacteria was detected based on disk diffusion method and the zone of inhibition was obtained for MRSA (12 mm), *S.aureus* 25923 (14 mm), *E. coli* ESBL$^+$: (10 mm), and *Klebsiella pneumoniae* (7 mm). Disk diffusion for P*seudomonas* and *Acinetobacter* demonstrated no inhibitory zones. Through Broth dilution method, MIC of cannabis extract on the bacteria was determined: *E.coli 25922*: 50*µg*/*ml, E.coli ESBL*$^+$:100 *µg*/*ml*, *S.aureus 25923*:25 *µg*/*ml*, *MRSA*: 50 *µg*/*ml*, *Pseudomona aeroginosa*ESBL$^+$> 100 *µg*/*ml*, *Pseudomonas*: 100 *µg*/*ml*, *Klebsiella pneumoniae*: 100 *µg*/*ml*, and *Acinetobacter baumannii*> 1000.

Conclusion: The maximum anti-microbial effect of the hydro-alcoholic extract of cannabis was seen for gram positive cocci, especially *S. aureus*, whereas non-fermentative gram negatives presented resistance to the extract. This extract had intermediate effect on Enterobacteriacae family. Cannabis components extracted through chemical analysis can perhaps be effective in treatment of nosocomial infections.

Keywords: Antibiotic resistance, Cannabis, Nosocomial Infections.

IJT 2014; 967-972

INTRODUCTION

Cannabis, also known as hashish, grass, marijuana, *Indica, Doga, Bang, and Ganja*, is the most commonly used illegal substance in the United States. Cannabis is a general term applied to the bioactive substances which are naturally or synthetically derived from *Cannabis sativa* [1].

Hemp, the plant that yields cannabinoid, is a diclinous plant. Gender identification is done on the basis of its flowers. Its male variety produces greater amounts of Cannabinoid. Generally, in more tropical environments the cannabinoid yield is greater. This is mainly due to reactions, such as alkylation, condensation, and the like, which require heat and temperature [2, 3]. The seeds of this plant have plenty of fat and double bands which make it inedible unless it is roasted and in Iran it is used in combination

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with roasted wheat seeds as a snack. Seeds of cannabis are used in soap and paint manufacturing industries and seed oil is of industrial use. The plant stems contain fiber and are used for making strawmat. The flowered branches of the plant contain resin which is rich in cannabinoids. Plant resins are located in trichome (hair-like structures found on the plant epidermis); thus, cannabinoids are located in plant resin [1].

Cannabinoids are monoterpenoid compounds which are obtained from *C. Sativa* extract and carboxylic acid metabolites, analogues, and transformation products. Δ^9 Tetra-hydrocannabinol (THC) is one of these cannabinoids which is considered the most active element of this group. Other compounds of this plant have little or no psychoactive effects.

Dronabinol is an oral Δ^9 THC which is applied as a Schedule II drug to the treatment of nausea and vomiting in patients and chemotherapy in the United States (rule) [3].

Cannabis Sativa contains 61 cannabinoid components and possesses 300 natural compounds, such as alcohol, steroids, glucoses, fatty acids, phenols, and trepans. The compounds present in natural cannabinoids existing in marijuana are diverse and their presence depends on the amount of rainfall, sunlight, temperature, genetic composition, altitude, and soil fertility. The highest concentration of components is present in the top of the plant which is flowering and declines in full blown flower, leaves, stem, seeds, and roots, respectively [3].

Hashish has long been known as a substance containing strong antibacterial agents with anti-*leishmanial* and anti-fungal properties [2, 4-7]. Unfortunately, the majority of research on hashish was performed at a time when chemistry as a science was not fully fledged and there is not much hard evidence on the anti-bacterial properties of this plant [6].

Currently, nosocomial infections and the propagation of varieties of microorganisms resistant to antimicrobial agents have turned to a critical issue in medicine. These infections are possibly generated by strains that develop resistance due to widespread excessive utilization of antibiotics. Accordingly, identification of new anti-microbial agents, particularly those with herbal basis is a substantial issue in medicine [6]. The aim of this study was to investigate the antibacterial property of *Cannabis sativa* on standard and resistant bacteria with multiple forms of resistance which are increasingly found in hospitals.

MATERIALS AND METHODS

Preparation of Clinical and Standard Strains

Initially, standard strains were collected from the laboratory of Vali-e-Asr Hospital, Arak, and identification tests were run for them. For examination of gram negative bacilli in terms of being ESBL+ (Extended-Spectrum Beta-lactamase Producing Bacteria), resistance to 3rdgeneration Cephalosporins test (cefotaxime, ceftriaxone, and ceftizoxime) and evaluation of the effect of their synergy with Co-amoxiclav was done. Hence, according to Clinical and Laboratory Standards Institute (CLSI) guidelines, 3rdgeneration cephalosporins were placed on Muller-Hinton agar at 2.5 to 3cm distances following double disk diffusion method to observe the effect of synergism. For evaluation of *S.aureus* in terms of being Methicillin-resistant Staphylococcus aureus (MRSA), oxacillin resistance test was performed.

Preparation of the Hydro-Alcoholic Extract of Cannabis through Liquid-Liquid Method

At first, 5g of cannabis, supplied from the Opioid Detection Police Unit and verified by the related lab, was dissolved in 10cc of alcohol and then in 100cc of distilled water. The solution was placed on a shaker with back and forth movements.

After two days of shaking, 10cc of 37% chlorhydric acid was added to the resulting solution and it was placed on an open flame so that its volume reduced to half and hashish was dissolved in the acid. After cooling the solution, 25% ammonium hydroxide was used to neutralize the acid phase so that pH of the solution reached 8.5-9. In order to create the aqueus alkali phase, chloroform and

isopropanol were added to the solution at a ratio of 80:20 and placed on the shaker for 20 minutes. For separating the remaining impurities, the solution was filtered by decanting and using filter paper. Then the low phase (aqueous phase) of this bi-phasic solution (aqueous-alkali) was separated and the remaining solution was placed in a boiling bain-marie (100°C) so that its solvent evaporated. After 4 hours, the obtained substance in the form of hashish oil was dissolved in methanol and transferred to a glass container which was placed in an incubator (45c) so that its methanol evaporated and hashish powder was obtained.

Figure 1. Separating the alkali phase from the aqueous phase of the base hashish solution after adding chloroform and isopropanol by decanting and using filter paper.

Determination of MIC

Initially based on disk diffusion method, the bacterial suspension adjusted to the 0.5 McFarland concentrations, it was transferred to Muller-Hinton agar plate. Then the disk, free of antibiotics, was placed on the surface of the plate by a sterilized forceps. After that, 20 microliter of the 25 µg/ml concentration was added to it and after 24 hours of incubation at 37°C, the area with growth inhibition around the disk was measured by a specific ruler (qualitative method).

The MIC's were determined for all strains that showed significant zones of inhibition. Initially the final concentration of 200 µg/ml cannabis was prepared in dimethyl-sulfoxide and MIC determination was performed using the two fold serial dilution method at a final concentration ranging from 100 mg/L to 1.56 mg/L(7 tubes). For negative control, a tube containing the culture media, bacteria, and dimethyl-sulfoxide was used while for positive control, a tube containing bacteria and culture media for every strain of the bacteria was incubated.

For preparation of microbial suspension from the fresh and young culture of the bacteria, 1 ml of each strain of the bacteria at 1 McFarland concentration was added to the tubes of each set so that the translucence of microbial suspension was eventually adjusted with 0.5 McFarland tubes which is equal to 108x 1.5 colony forming unit per ml (cfu/ml).

All of the culture media were incubated for 24 hours at 37°C. After this period, all of the tubes were evaluated in terms of translucency. The first tube without translucency (inhibition of bacterial growth) was considered as MIC.

RESULTS

The sensitivity of the bacteria was evaluated through disk diffusion (see Table 1 for the results). Disk diffusion method was also utilized for *Pseudomonas* and *Acinetobacter* but did not indicate growth inhibition zones.

Table 2 shows the results of MIC test after the 24-hour period of incubation at 37°C.

Table 1. Disk diffusion results for bacterial sensitivity to cannabis extract.

Bacteria	Inhibition zone diameter (mm)
MRSA	12
S.aureus 25923	14
E.coli ESBL +	10
Klebsiella pneumoniae	7

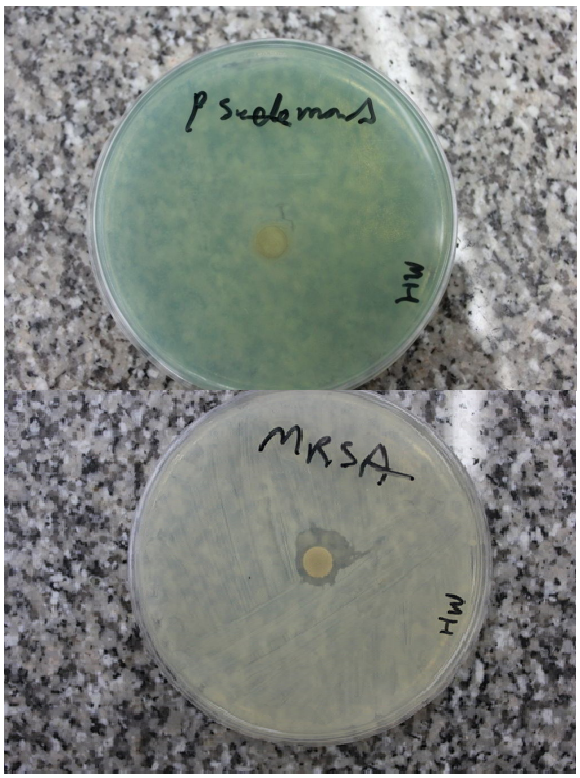

Figure 2. The formation of MRSA growth inhibition zone and its absence aroundPseudomonas.

Table 2. The results of MIC (µg/ml) test of cannabis extract after 24 hour incubation period.

Bacteria	MIC (µg/ml)
E. coli 25922	50
E. coli ESBL +	100
S.aureus 25923	25
MRSA	50
Pseudomona aeroginosa ESBL+	>100
Pseudomonas	100
Klebsiella pneumoniae	100
Acinetobacter baumannii	>100

The smaller MIC values indicate higher anti-microbial effects. In the case of *Pseudomonas* and *Acinetobacter baumannii*, for investigating if MIC were higher or not, serial dilution method as explained earlier, but at 250, 500, 1000, and 2000 µg/ml concentrations was run. However, their growth inhibition did not take place even at higher concentrations. It should also be noted that all of the five strains of each type of the clinical bacteria generated the same results.

DISCUSSION

Biologic compounds with herbal basis are an important part of drug therapy and in many instances, have come to receive greater attention as better alternatives due to their ease of access, reduced side effects and prices in comparison with synthetic drugs [8]. The antimicrobial activity of various resins of cannabis can depend on geographic location, climate, plant genetic traits, and other ecologic factors [9].

In the present study, the greatest anti-microbial effect of hashish extract was observed on *S.aureus*25923, whereas it practically had no significant effects on *Pseudomonas* and *Acinetobacter baumannii* at the aforementioned doses. In general, the lowest MIC value was observed for *S.aureus* (MIC: 25) and after that for *E.coli* 25922 and MRSA (MIC: 50), *Klebsiella pneumoniae* , *Pseudomona aeroginosa*ESBL^{+} (MIC:100) and *Acinetobacter* and *Pseudomonas* ESBL^{+} which remained resistant even at high doses, respectively.

In a study done by Nissen*et al*. (2001) entitled"Characterization and antimicrobial activity of essential oils of industrial hemp varieties (*Cannabis sativa L*.)", different oils of 3 varieties of Cannabis Sativa were extracted through distillation, gas chromatography (GC), and spectrometry and their anti-microbial effects on three classes of microorganisms, including gram positives, gram negatives, and fermentives, were examined. The results showed that the industrial oil of cannabis significantly inhibited the growth of microbes. This property depended on the variety of the plant and the number of planting times so that the greatest anti-microbial property was observed in *Futura* variety [10]. Since in the present study, we used the illegal cannabis extract confiscated by narcotic police forces, it was not possible to examine the effect of plant variety and the number of planting times and only the hydro-alcoholic extract of the plant was examined, a comprehensive comparison cannot be made between the two studies. As it was mentioned, Nissen *et al*'s used the industrial oils of the plant which followed a different extraction method compared to our study. However, antibacterial properties of the cannabis were confirmed by both studies.

Borchardt *et al*. (2008) carried out a study on the anti-bacterial effects of the

leaves, root, and stem hydro-alcoholic extracts of 336 plant species, native to Minnesota and Wisconsin, US, on *S.aureus*, *E. coli*, *Pseudomona aeroginosa*, and *Candida albicans*. This study employed the dried extract of the plants, the leaves and roots in the case of *Cannabis Sativa*, through disk diffusion method. The results demonstrated inhibitory effects of leaves and root extract of *cannabis sativa* on *S.aureus* (inhibition zone: 25mm). Also, cannabidol was revealed to be effective against fermentives and gram positives and a strong relationship was observed between the level of cannabidiolic acid and anti-microbial activity. This study demonstrated that *cannabis sativa* growing in higher latitudes has a higher level of Cannabidol: Δ^9 THC and, as a result, its antimicrobial activity is more [11]. In the present study, the plant extract effectively inhibited the growth of MRSA and *S.aureus* and had a relatively good inhibitory effect on *E.coli*. This can be due to the difference in *E.coli* strains, the plant species or the part of plant used for extraction, and the geographic location in which the plant grows. Since in the study conducted in Minnesota, the extracted parts were used for their anti-microbial property, a comparison of the concentrations is not plausible.

Sumthong and Verpoorte (2007) conducted a research to evaluate the anti-microbial activity of the components of *Cannabis sativa* alcoholic extract (CHCL3-MEOH) (1:1) through disk diffusion method. They placed 2mg of the extract on sterilized disks and examined its effect on *Bacillus subtilis* and *E.coli* (LMD 72.2). It turned out that *Cannabis sativa* inhibits the growth of both bacteria though the greatest inhibitory effect was from the flower extract (CHCL3-MEOH) of *Cannabis sativa* on *Bacillus subtilis* (Mean inhibitory zone: 23ml), while this value for *E.coli* was 11 mm. This study was done on the alcoholic extract of cannabis flowers with a concentration four times more than that of the present study through disk diffusion method [12]. Since the inhibition zone was quite close in both studies, it can be said that this difference in concentration for the formation of growth inhibition zone can be due to different geographic location of plant growth, type of solvent used for extraction, and *E.coli* strain.

Vanklingern also investigated the anti-microbial activity of two cannabis components, Δ^9 THC and Cannabidol (CBD) provided from the Opioid Center of the United States, on *S.aureus* through broth dilution method and reported the MIC test of both components with concentrations between 1 to 5 µg/ml. In culture media containing 4% serum and 5% blood, MIC increased and reached to 50 µg/ml. Gram negatives were also resistant to these components. Similar to the present study, the two components of cannabis were effective against *S.aureus* [7]. American Type Culture Collection (ATCC) of *S.aureus* in this study was 6538 while it was 25923 in the present study. There was also a significant difference between MIC of the Vanklingern study and the present study which can be due to differences in strains and it can justify the increased microbial resistance to cannabis in the past years.

Moreover, separation of cannabis components can increase their anti-microbial effect on *S.aureus*. In the present study, cannabis extract had inhibitory effects on *Klebsiella pneumoniae* and *E.coli* at higher concentrations, whereas in the Vanklingern study intestinal gram negatives were resistant to these components. This can be due to the effect of other components of cannabis on intestinal gram negatives bacteria [7].

Appendino conducted a study on the anti-microbial activity of cannabinoid analogues of Cannabis Sativa and investigated all the 5 components of cannabis plant, including CBC (cannabichromene), CBG (cannabigerol), CBD (cannabidiol), THC (Δ9-Tetrahydrocannabinol , and CBN (cannabinol), which showed anti-bacterial activity with MIC values ranging from 0.5 to 2µg/ml (8). These components were particularly effective in the case of epidemic clinical MRSA in hospitals of England. The obtained MIC value for MRSA fluctuated between 0.5 and 64 µg/ml which is a lot less compared to MIC values for antibiotics like erythromycin (more than 128) which was used as control [6].Since this study investigated the effect of cannabinoid

analogues, its results cannot be compared quantitatively to the findings of the present study; however, the common ground between them is their focus on the anti-bacterial effect of cannabis, especially its strong inhibitory effect on clinical MRSA.

Nevertheless, to date, no major studies have been done on the effect of cannabis on *Acinetobacter baumannii* and *Pseudomona* $ESBL^{+}$. Genetic study of these strains have shown that they are extensively drug-resistant (XDR) resistant and resistance to cephalosporins, aminoglycosides, kinolons, and carbapenems is prevalent. Moreover, they have a specific characteristic in regard to efflux pump that do not allow entrance of anti-microbial agents into their cells. Thus, if such agents enter the bacteria, they are dispelled actively by ATP-powered pumps. Apparently, resistance to cannabis can be justified by this mechanism.

Klebsiella pneumoniae which was included in this study is considered MDR and it is resistant to a wide range of antibiotics. The greatest antimicrobial effect of the hydro-alcoholic extracts of cannabis was observed on gram positive cocci, especially *S.aureus*, while non-fermentative gram negatives were resistant to it. The extract had an intermediate effect on intestinal gram negatives. This extract can probably be applied for the treatment of infections by separating its components through chemical analysis.

CONCLUSION

It can be concluded that, gram positive bacteria were more susceptible than gram negative bacteria to cannabis extract. More extensive studies on different organs of this plant through using different biochemical analyses and extraction methods to discover components with antibacterial properties, such as acetate extraction method, as well as using different varieties of Cannabis sativa from different geographic regions, are recommended.

ACKNOWLEDGMENT

This article has been extracted from Dr Tahereh Haji-Mir thesis. No financial support has been received by the authors from any organization.

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