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Full Length Research Paper

'Extraction of cannabinoids from cannabis sativa L plant and its potential antimicrobial activity'

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Drug resistance of microorganisms is a big threat for the human health. In order to overcome this problem an alternative potential treatment is the use of herbal medicines. Plants are not only considered as dietery supplements to living organisms but also tradionally used for treating many diseases and no sign of drug resistance has been reported till now. The plant leaves were identified as *Cannabis sativa L.* The cannabinoids were extracted by aqueous extraction found a total yield of 3.8g while as acetone extract 4.8g. The protein content in crude extract of *Cannabis sativa L.* for aqueous extract was found 112µg/ml and for acetone extracts 160µg/ml. The molecular weight of protein by SDS PGAGE was found to be 70KDa. The HPLC intension percentage for aqueous extract was 11 while for acetone extract it was found 25. The acetone extract exhibited higher antimicrobial activity by showing zone of inhibition against bacteria *Pseudomona aeruginosa*, *Vibro cholerae* and fungi *Cryptococcus neoforms, Candida albicans*. The cannabiniods extracts showed the antioxidant activity by changing to yellow colour.

Key words: Cannabinoids, Plant extracts, Crude extracts, Anibacterial activity, Antifungal activity, antioxidant activity.

INTRODUCTION

Marijuana (*Cannabis sativa*) has long been known to contain antibacterial cannabinoids, whose potential to address antibiotic resistance has not yet been investigated. All five major cannabinoids such as cannabidiol, cannabichromene, cannabigerol, Δ9-tetrahydrocannabinol and cannabinol showed potent activity against a variety of methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Appendino et al., 2008). The drug is known all over the world for its good euphoric and medicinal properties. The plant and its preparations have been used for its antibacterial studies on the hemp grown in different parts of the world (Kreji, 1958). The substance possess good antimicrobial activity against *Mycobacterium tuberculosis*, Gram-negative bacteria of the Coli-typhus group, *Pseudomous aeruginosa* and *Proteus Vulgar,* acid-fast bacteria, yeast-like fungi, filamentous fungi and a dermatophyte (Turner et al. 1981; Wasim et al., 1995). The known sterol beta-sitosterol-3-O-beta-d-glucopyranosyl-6'-acetate displayed significant antibacterial, antifungal activities and strong antileishmanial activity (Radwan et al., 2009). The activity was exceptionally high when tested with the multidrug-resistant SA-1199B (Roth et al., (2002). The minimum inhibiting concentrations, antibactericidal acitvity of delta9-tetrahydrocannabinol and cannabidiol for *Staphylococci* and *Streptococci* in broth are in the range of 1-5 µg/ml. In media containing 4% serum or 5% blood the antibacterial activity is strongly increased minimal inhibitory concentration to 50µg/mL (Klingeren and Ham, 1976). Cannanbinoids had potent antileishmanial activity and effective to killing *Candida albicans*, a fungus that cause oral and genital infections (Whittakar et al., 2004). The both herpes simplex virus type 1 and herpes simplex virus type 2 were exposed to various concentrations of delta-9-tetrahydrocannabinol showed a plaque assay utilizing confluent monkey cells that had possible mechanisms for antiviral activity and that this activity is modulated by the presence of serum proteins (Lancz et al., 1991; Blevein et al. 1980). The cannabinoids showed the oxidative stress on MDA-MB-231 cells and lead reactive oxygen

species (ROS) formation in MDA-MB-231 cells which depends upon dose and time (Ligresti et al., 2006).

MATERIALS AND METHOD

Collection and preservation of specimen:

The plant leaves of *Cannabis sativa* were collected from Kolli hills, Tamil Nadu and were taken to the laboratory; subsequently they were preserved in 10% neutralized formalin at low temperature.

Extraction of crude toxin:

The aqueous extract of sample was prepared by squeezing the sand free specimens in triple distilled water (Guzman, 2003). The resultant solution was filtered and dialyzed by using sigma dialysis membrane-500 (Av Flat width-24.26 mm, Av. Diameter-14.3mm and capacity approx-1.61ml/cm) against D-glucose to remove the excess water. The supernatant so obtained was stored at 4°C and then used as crude aqueous extract.

Acetone extraction

The sample was dried in air for 2 days and after complete drying, crushed in the form of fine powder then 10g sample was added into 200ml of acetone, covered and allowed to stand for 5 hours (Teske et al., 2002). The solvent was then removed after squeezing the sample and filtered through Whatman filter paper No1. The solvent was evaporated at low pressure by using a Buchi Rotavapor R-200 at 4$^{\mathbf{0}}$C and stored in refrigerator to use as crude acetone extracts.

Purification of Crude Protein

Purification of the crude extract was carried out by using DEAE cellulose anion exchange chromatography equilibrated with 10mM Tris-HCl buffer pH 8.3 (Teske et al., 2002). The 15ml of crude extract was eluted with 10mM Tris-HCl buffer pH 8.3 at the flow rate of 20ml/h. A linear gradient consisting of 50ml of 10mM Tris-HCl buffer pH 8.3 and 50ml of the same buffer with 0.3M NaCl. The 5ml of fractions elute was collected and absorbance measure at 280nm.

Protein Estimation

Protein estimation of *Cannabis sativa L* extracts was done as described by Lowry et al. (1951) by using Bovine serum albumin standard and absorbance was read at 650nm under spectrophotometer.

Characterization of protein:

Sodium Dodecyl Sulphate–PAGE

One dimension sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the modified method of Lammelli et al., (1970). The molecular weight of the protein determined by staining the protein with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 hours and destained with 10% methanol, 25% acetic acid solution for 12 hours.

HPLC Chromatography

The column used here was “Normal Phase” having Cartridge 8 x 100 mm Bondapak C18 and Solvent contains 0.5% phosphoric acid in 40% aqueous acetone (Debruyne *et al*., 1999). In HPLC, narrow columns with internal diameters 2-80 mm were used. The solvents were filtered through fine membranes (0.4-0.5 micron) and all solutions were injected into the Water’s instrument comprises of three main components: the injector (located on the top right-hand corner of the console), the solvent delivery system (right-hand side) and the UV detector (left-hand side).

Antimicrobial activity

Antibacterial activity

The Mueller Hinton agar medium was prepared (Wasim et al., 1995), sterilized and poured in Petri plates, after solidification were inoculated with two different species of bacteria viz *Pseudomonas aeruginosa* and *Vibrio cholerae*. The aqueous extract and acetone extract samples of cannabinoid were sterilized by passing each through a 0.22 µm Millipore GV filter (Millipore, USA). Round paper discs with a radius of 0.8cm were dipped into (5µg/ml and 10µg/ml) each sample extract and placed in the center of inoculated petri dishes and incubated at overnight at 37^{0}C then the inhibition zone around the disc was measured.

Antifungal activity

The Mueller Hinton agar medium was prepared (Wasim et al., 1995), sterilized and poured in peteripltes after

Table.1. Estimation of cannabinod protein from acetone extract and aqueous extract by following Lowry etal (1958) method using the bovine serum albumin as standard

S.No	Type of Extract	Conc of protein (mg/ml)
1.	Acetone	1.6mg/ml
2	Aqueous	0.6mg/ml

Note: Where mg means milligram, ml means milliter

solidification were inoculated with two different species of fungi viz *Cryptococcus neoforms, Aspergillus niger, Candida albicans*. The aqueous extract and acetone extract samples of cannabinoid were sterilized by passing each through a 0.22 µm Millipore GV filter (Millipore, USA). Round paper discs with a radius of 0.8cm were dipped into (5µg/ml and 10µg/ml) each sample extract and placed in the center of inoculated petri dishes and allowed to grow for 48 hours at 28^0C then the inhibition zone around the disc was measured.

Antioxidant assay

The concentrate extract was prepared by following Radwan *et al.,* 2009 with 0.1mg/ml of crude extract and 50µl of each extract was loaded into U bottom 96 well plates. 50µl of 0.11% DPPH was added to it. Ascorbic acid (0.1 mg/ml) was used as the positive control and sterile water was used as negative control. The plate was incubated at dark for 30 minutes for the colour change. Development of purple colour to yellow colour within 30 minutes indicates positive result

RESULT

Identification of Leaves

The present study deals with to study the antimicrobial activities of herbal plant *Cannabis sativa L*. The sample collected from Kolli hills, Tamil Nadu, was identified based on the taxonomical characters. Kingdom-Plantae, Class-Magnoliopsida, Order-Rosales, Family-Cannabaceae, Genus-Cannabis, Species-*Sativa L*

Extraction of Protein

Acetone extract of plant leaf sample *Cannabis sativa L* yielded a total amount of 3.2g of crude extract from 500g of sample. Similarly from aqueous extract at a total amount of 4. 8g of crude extract was yielded.

Protein Content

The optical density values of aqueous extract obtained 0.530nm which corresponded to 0.6mg/ml. While in acetone extract the optical density found was 0.676nm which corresponds to 1.6mg/ml (Table 1).

DEAE Chromatography

The optical density value of cannabinoid of aqueous extract at 280nm was 1.530nm and for acetone extract the optical density of cannabinoid was 1.745nm.

Molecular weight of Protein

On 12% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the crude protein toxins yielded 3 bands in aqueous extract and 5 bands in acetone extract of *Cannabis sativa L* with one well having a defined band of 70 KDa (Figure 1).

HPLC chromatography

Under the high performance liquid chromatography it was analyzed that in the acetone extract of retention time of 3.27min the percentage intension was found to be 11 for aqueous extract and 25 for acetone extract. In the aqueous extract the retention time of 4.75min, the percentage intension was found to be 32 for aqueous and 30 for acetone extract.

Antibacterial activity

The crude aqueous extract and acetone extract at different concentration of 5µg/ml and 10µg/ml showed good antibacterial activity against two species of bacteria such as *Pseudomonas aeruginosa* and *Vibrio cholera* are presented in Table 2.

Antifungal activity

The crude aqueous extract and acetone extract at different concentration of 5µg/ml and 10µg/ml showed

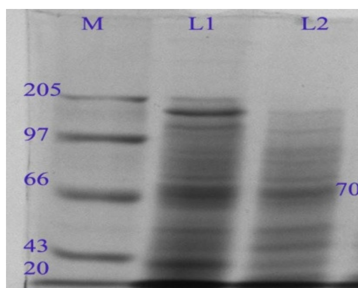

Figure 1. Determination of molecular weight of proteases by sodium dodecyl sulphate agarose gel electrophoresis.
M— Molecular marker mass standards: phosphorylase b (205 kDa), tyrosine (97kDa), acid phosphate (66kDa), glutamic dehydrogenase (43 kDa) and aldolase (20 kDa)
L1—Acetone extract of Cannabinoids showed 5 bands with distinct band of 70KDa.
L2—Aqueous extract of Cannabinoids showed 3 bands with distinct band of 70KDa

Table 2. The antibacterial activity of aqueous exrtract and acetone extract of cannabinoid against Pseudomonas aeruginosa and Vibrio cholera showed a zone of inhibition when used in different concentrations.

S.no	Fungal culture	Aqueous		Acetone	
		5µg/ml	10µg/ml	5µg/ml	10µg/ml
1	*Cryptococcus neoforms*	4mm	8mm	5mm	10mm
2	*Candida albicans*	4mm	8mm	6mm	12mm

Where µg indicates the concentration in micrograms, mm means in millimeter the zone inhibition

Table 3. The antibacterial activity of aqueous exrtract and acetone extract of cannabinoid against Cryptococcus neoforms and Candida albicans showed a zone of inhibition when used in different concentartions

Sl.no	Bacterial culture	Aqueous		Acetone	
		5µg/ml	10µg/ml	5µg/ml	10µg/ml
1	*Pseudomonas aeruginosa*	3mm	6mm	4mm	8mm
2	*Vibrio cholerae*	4mm	8mm	5mm	10mm

Note: Where µg indicates the concentration in micrograms, mm means in millimeter the zone inhibition

good antifungal activity against 2 species of fungi such as *Cryptococcus neoforms, Candida albicans* are presented in Table.3

Antioxidant assay

The DPPH reaction both aqueous and acetone extracts changed the DPPH molecules to yellow colour which indicated the presence of antioxidant molecules are shown in Figure 2

DISCUSSION

Drug resistance is a biggest threat to human health this occurs due to various factors to overcome this problem an alternative treatment has been chocen. The plant materials are important to cobat serious disease in the

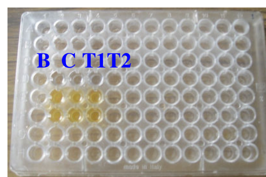

Figure 2. Antioxidant assay of the aqueous extract and acetone extract of cannabinoid showed the colour change to yellow that indicates the antioxident activity where B is the blank controls, C is the positive control, T1 is the test sample of aqueous ectract of cannabinoid and T2 is the test sample of acetone extract of cannabinoid

world. The antimicrobial properties of the plants are being increasing reported from different parts of world. Activity was remarkably tolerant to the nature of the prenyl moiety by taking together these observations suggest that the prenyl moiety of cannabinoids act good antimicrobial activity (Appendino, *et al* 2008). The acidic fraction from the ethanolic extract and 2% sodium hydroxide extract from Cannabis *sativa* L exhibited activity against both Gram-positive and Gram-negative bacteria and also against the fungi (Wasim et al., 199; Radwan et al., 2009). The earlier observation showed that cannanbinoids have potent activity against two strains responsible for MRSA, namely EMRSA-15 and EMRSA-16. These two strains were the main culprits for MRSA epidemics in hospitals at UK (Roth *et al*., 2002). Here Cannanbinoids were very effective to killing *pseudomonas aeruginosa, vibro cholerae, Cryptococcus neoforms*, *Candida albicans*, a fungi that causes aspergillosis and oral and genital infections (Whittakar et al., 2004). Antibacterial activity of Cannabichromene and its isomers showed strong antimicrobial activity against gram-positive, gram-negative, acid-fast bacteria, yeast-like fungi, filamentous fungi and a dermatophyte (Turner et al. 1981). The minimum inhibiting concentrations of aqueous extract and acetone extract of cannabinoid against *Pseudomonas aeruginosa, Vibro cholerae, Cryptococcus neoforms*, *Candida albicans* were in the range of 5µg/ml and 10µg/ml. In the same range both compounds showed bactericidal and fungicidal activityies. The media containing 4% serum or 5% blood the antibacterial activity is strongly increased minimal inhibitory concentration 50µg/ml (Klingeren and Ham, 1976). DPPH is a useful reagent for investigating the free redical scavenging activities of compounds. The actone and aqueous extracts were reduced the stable redical DPPH to yellow coloured diphenyl picrylhydrazine thus indicates that cannabinoids can be used potential antioxidant agent (Ligresti et al., 2006).

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