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Antimicrobial activity of *Cannabis sativa* extracts on Lancefield Group A *Streptococcus* species associated with streptococcal pharyngitis (strep throat)

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Abstract

Cannabis sativa is a herb with a rich diversity of active ingredients with various pharmacological properties ranging from psychoactive, sedative, analgesic, anti-inflammatory and antimicrobial activities. This study was undertaken to evaluate the efficacy of leaf extracts of *Cannabis sativa* on inhibiting the growth of Lancefield Group A *Streptococcus* sp responsible for streptococcal pharyngitis also known as "strep throat". The active ingredients of *Cannabis sativa* were extracted using water and methanol in a soxhlet apparatus and measured using standard protocols. 10 Group A *Streptococcus* spp. were isolated from clinical cases of streptococcal pharyngitis. The susceptibility of these isolates to the methanolic extract of *Cannabis sativa* was evaluated using the Kiby-Bauer agar-well diffusion assay technique and tube dilution method. The antimicrobial inhibitory properties of the extracts were compared to three common antibiotics used in the treatment of strep throat (penicillin, amoxicillin and chloramphenicol). Results obtained shows the presence of bioactive compounds including; alkaloids, flavonoids, cardiac glycosides, phenols, terpenes, resins and steroids. These phytochemicals exerted antimicrobial activity against *Streptococcus* sp, resulting in zones of inhibitions between 18.80-22.80 mm against the test organisms, comparable to the zones obtained from commercially available β-lactam antibiotics. Extracts of cannabis out-performed chloramphenicol in the inhibition of the test organism, producing larger zones. Tube dilution assays of the extracts gave a Minimum Inhibitory Concentration (MIC) of 20 mg/ml and Minimum Bactericidal Concentration (MBC) of 30 mg/ml, all comparable to the commercial antibiotics. Results of this study have highlighted the potential of cannabis extracts to control Lancefield Group A *Streptococcus* sp which are causative agents of pharyngitis.

Keywords: *Cannabis, Antimicrobial activity, Strep throat, Streptococcus, Lancefield Group A.*

1. Introduction

Pharyngitis/tonsillitis is an inflammation of the posterior pharynx and tonsils, caused by a variety of microorganisms mainly viruses and bacteria (Anjos *et al.*, 2014). *Streptococcus pyogenes,* a Lancefield Group A β-hemolytic streptococci is one of the most implicated aetiological agent in this disease condition (Ralph and

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Carapetis, 2013). Pharyngitis is characterized mainly by sore throat and fever with most cases of pharyngitis resolving without any medical treatment / intervention. However, in few cases, the infection can become invasive, resulting in complications including rheumatic fever (RF), autoimmune post-streptococcal sequelae and glomerulonephritis (Ralph and Carapetis, 2013; and Wessels, 2011) which can have more debilitating effects. Because of the possibility of a benign streptococci infection to progress to more invasive forms as highlighted above, pharyngitis infections caused by drug-resistant strains of *Streptococcus* sp. are important from the public health point of view as these can cause other ailments that may not be easily treated using conventional antibiotics. Presently, there is an increase in the occurrence of multi-drug resistant *Streptococcus* sp. in clinical cases, thus underpinning the need for close monitoring and the introduction of alternative approaches for the treatment and management of pharyngitis cases (Chen *et al.*, 2011).

Many of the medicinal attributes of plants are determined by the bioactive components of their phytochemistry (Gill, 1988) which have both antimicrobial, anti-inflammatory and allied properties. Thus, some medicinal plants are applied in the treatment of disease conditions, with about 25% of modern medicine being composed of one or more extracts of plants and / or their synthetic analogues (De Silva, 2005). Furthermore, medicinal plants have been known to enhance the effects of antibiotics, giving a synergistic effect which is higher than the inhibition of microorganisms recorded upon the use of antibiotics alone (Chakraborty *et al.*, 2018). *Cannabis sativa* colloquially as 'Indian hemp' is one of the most recognizable and widespread plants on earth and has been cultivated for its diverse uses including as a source of oils, fiber, medicine, food and as a psychoactive substance (Nagy *et al.*,. 2019). It is a source of new biomaterials and biofuels (Bonini *et al.*, 2018). It is a herbaceous annual plant cultivated worldwide. It is originally native to Central Asia and has found use since ancient times for both recreational, food, medical and spiritual purposes (Piluzza *et al.*, 2013). Cannabis has a variety of bioactive compounds that make up its phytochemistry including; alkaloids, flavonoids, cannabinoids and terpenoids (Andre *et al.*, 2016). These bioactive compounds are responsible for its attributes. The plant is used in the treatment of sleep disorders especially in individuals with chronic pain or post-traumatic stress disorder (Babson *et al.*, 2017), in the treatment of gut diseases, such as gastrointestinal pain, gastroenteritis, and diarrhoea (Couch *et al.*, 2018) amongst others.

Apart from the use of extracts of cannabis in the treatment of disease conditions, they have been shown to exert an antimicrobial activity, inhibiting the growth and proliferation of various microbial species. A study by Chakraborty *et al.* (2018) on the antimicrobial activity of *Cannabis sativa* against Methicillin-resistant *Staphylococcus aureus* (MRSA) showed that extracts of the plant were able to inhibit MRSA which is notorious as being difficult to treat due to its multi-drug resistant nature. This showed the potential of the plant extract to be employed in the control of infections by multi-drug resistant strains of pathogens as demonstrated in our previous study on the antimicrobial activity of extracts of tobacco leaf (*Nicotiana tabacum*) and its ground snuff against *Streptococcus pyogenes* and Candida albicans (Anumudu *et al.*, 2019). Numerous other studies have highlighted the activity of the cannabis extracts on various pathogens including *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (Ali *et al.*, 2012). Literature on the efficacy of the plant extract against Lancefield Group A β-hemolytic *Streptococcus* sp. is limited. Thus, this study is undertaken to assay the ability of the extracts to inhibit these organisms and compare their activity to commercially available antibiotics employed in the treatment of pharyngitis / tonsillitis.

2. Materials and methods

2.1. Source of test organism

Isolates of *Streptococcus pyogenes* were obtained from stock cultures isolated from clinical patients in the Imo State University Owerri teaching hospital diagnosed with strep throat using sterile swabs. Swabs obtained were cultured on trypticase soy agar supplemented with 5% sheep blood and incubated for 24 h at 37 °C. After overnight incubation, resulting colonies were subcultured to obtain pure cultures. These were utilized for further biochemical screening including bacitracin susceptibility testing and Lancefield grouping for the definitive identification of Lancefield Group A *Streptococcus* sp.

2.2. Identification of isolates

For confirmation of bacteria samples, beta haemolytic colonies on trypticase soy agar supplemented with 5% sheep blood which results in the lyses of the sheep blood in the agar and the clearing of the blood surrounding the colonies were selected. These were subjected to Gram staining and catalase test using hydrogen peroxide for presumptive identification of *Streptococcus.*

Bacitracin sensitivity test was undertaken on Muller-Hinton agar. Isolates were inoculated heavily over the surface of the agar plate. 0.04 units of bacitracin disks (Merck, Germany) were placed on the plates and incubated overnight at 37 °C according to standard procedure (Zige and Anumudu, 2019). After overnight

incubation, resulting zones of inhibition were measured using calibrated callipers. Zones of inhibition ≥10 mm were considered as sensitive.

The Lancefield agglutination assay was undertaken using the STREP test kit (Plasmatec, UK) following the protocol stipulated by the manufacturers. A positive Lancefield test is indicated by a visible agglutination of latex particle and this gives confirmatory identification of *Streptococcus pyogenes.*

2.3. Preparation of leaf extracts

Fresh leaves of *Cannabis sativa* were collected from the Imo State University research botanic garden in Owerri, Nigeria. Identification of the plant was made by the Plant Science and Biotechnology Department of the Imo State University using a leaf sample which was collected together with stem and seeds, dried and pressed onto a herbarium sheet.

After collection, the leaves were washed with distilled water, blotted dry and placed in a drying oven maintained at 60 °C with circulating air for 48 h. The dried leaves were crushed using a laboratory homogenizer. 100 g of the crushed leaves was transferred to a soxhlet apparatus and 500 ml of methanol was used to extract the active components at 80 °C. After soxhlet extraction, the solvent was removed using a rotary vacuum extractor. The obtained residues were dissolved with Dimethyl sulfoxide according to the method of Anumudu *et al. (*2019) and filtered using Whatman syringe driven filter with pore size 0.2 μm. Obtained extracts were stored in the refrigerator until needed for assay.

2.4. Phytochemical characterization of extracts

The phytochemical components of *Cannabis sativa* were evaluated by determination of the presence of bioactive compounds including alkaloids, flavonoids, glycosides, phenol, tannins, resins, terpenes and steroids using standard phytochemical procedures as described by the association of official analytical chemists with some modifications (Ates *et al.,* 2003).

2.5. Antimicrobial assay

Antimicrobial activity of the leave extracts of *Cannabis sativa* was evaluated by the agar diffusion assay (Kirby-Bauer method) using Mueller Hinton Agar (MHA). Firstly, the concentration of the test organism (*Streptococcus pyogenes*) in broth culture was adjusted to the MacFarlands standard by diluting with normal saline and measuring absorbance at 600 nm using a UV / Vis spectrophotometer. After standardization of the organism, 100 μl inoculum was introduced and uniformly spread onto the agar plate using a sterile bent glass rod and allowed to dry. A 6 mm well capable of retaining 60 μl was made on the agar plate using a sterile cork borer. Using a calibrated pipette, 50 μl of the extract was introduced into the wells. Plates were incubated for 36 h at 37 °C. After incubation, bacteria sensitivity to the extract was evaluated by measuring resulting zones of inhibition around each well using a calibrated ruler. All analysis was carried out in triplicates.

The performance of the methanol extracts of *Cannabis sativa* was compared to the inhibition of *Streptococcus* sp. by two common antibiotics in the β-lactam family (Penicillin G and Amoxicillin) and a phenicol (chloramphenicol) usually administered in the treatment of strep throat. This was done using commercial antibiotics discs on MHA inoculated with the test organisms. All plates were incubated for 36 h at 37 °C. After incubation, resulting zones of inhibition were measured using a calibrated ruler.

2.6. Determination of minimum inhibitory and bactericidal concentration

The Minimum Inhibitory Concentration (MIC) of the extract was evaluated by the tube dilution method. 100 μl of the inoculum adjusted to the MacFarlands standard was inoculated onto individual test tubes containing 9 ml of pre-sterilized nutrient broth. To each of the tubes, 1 ml of a reducing concentration gradient of the extract is added. This was mixed uniformly, the initial absorbance at time zero was taken. All tubes were incubated overnight. After overnight incubation, change in absorbance / development of turbidity was determined by visual examination and the use of a spectrophotometer. The MIC was taken as the lowest concentration of extract added to a tube which showed no change in turbidity / cloudiness thus indicating inhibition of growth.

An inoculum was taken from clear tubes without turbidity and transferred to fresh agar plate using an inoculating loop. The inoculated agar plate was incubated overnight at 37 °C. the plate with the lowest concentration of extract which showed no growth / colony formation is considered the Minimum Bactericidal Concentration (MBC), thus indicating complete cell death.

2.7. Result analysis

All experiments were carried out in triplicates. Mean values and standard deviation were obtained for the diameter of inhibition zones, and geometric mean MICs were calculated. The results were expressed as mean values ± SD or as geometric means.

3. Results and discussion

3.1. Test organisms

The methanol extracts of the leaves and seeds of *Cannabis sativa* were screened for their antibacterial activity against Group A *Streptococcus* sp isolated from clinical patients diagnosed with strep throat. The test organisms were Gram-positive cocci in chains or pairs, beta haemolytic and catalase-negative. Upon subjection to bacitracin assay, the organism was highly sensitive and gave a positive Lancefield agglutination assay result, confirming its identity as a Group A *Streptococcus* sp. A total of 10 Group A *Streptococcus* sp. were obtained and used for this study.

3.2. Phytochemical characteristics

Phytochemical analysis of the bioactive components of *Cannabis* shows the presence of these compounds which may be responsible for the antimicrobial activity of the extracts and may similarly be responsible for the aroma, addictive and hallucinogenic nature of the plant. The phytochemical components are presented in Table 1.

Table 1: Phytochemical analysis of methanolic extracts of *Cannabis sativa*

Phytochemical	Color	Presence
Alkaloids	Orange	+
Saponins	-	-
Flavonoids	Light yellow	+
Tannins	-	-
Cardiac glycosides	Reddish brown	+
Phenols	Bluish black	+
Terpenes	Reddish brown	+
Resins	Violet	+
Steroids	Brown	+

Note: + positive; and – negative.

The results shown above indicate that *Cannabis sativa* is rich in phytochemicals which confers on it unique attributes. The results obtained in this study is in tandem with the findings of Chakraborty *et al.* (2018) in their investigation of the antimicrobial activity of *Cannabis sativa* against MRSA. The phytochemical profile of *Cannabis sativa* shows that the plant can be a source of non-psychoactive bioactive cannabinoids, sesquiterpenes and flavonoid glycosides which have important pharmaceutical applications. Previous research (Nagy *et al.*, 2019) has revealed that the plant contains more than 84 volatile components of with the highest proportion being sesquiterpene hydrocarbons (57.1-62.8%) followed by cannabinoids (11.0-29.3%) and oxygenated sesquiterpenes (7.8-14.8%). These essential oils have been shown to have potent antimicrobial activity (Barrero *et al.*, 2005) and may be responsible for the inhibition of growth of the test organisms recorded in this study.

3.3. Antimicrobial susceptibility

10 isolates of Group A *Streptococcus* sp. were isolated from clinical patients. These were used to evaluate the antimicrobial activity of the cannabis extracts and compare them to commercial antibiotics. Table 2. below shows the produced zones of inhibition (mm) and susceptibility of the different isolates using the well in agar and disk diffusion assay of the Kirby-Bauer method.

Table 2: Zones of inhibition of *Cannabis* extracts and antibiotics against *Streptococcus* sp.

Isolates	Mean values ± SD (mm)			
	Chloramphenicol (30 μg)	Penicillin G (10 μg)	Amoxicillin (20 μg)	*Cannabis* extract (30 mg)
1	18.30 ± 2.2	23.32 ± 2.3	22.76 ± 2.9	19.40 ± 1.6
2	18.32 ± 3.2	22.42 ± 2.8	19.88 ± 2.6	19.18 ± 2.2
3	18.18 ± 2.2	21.88 ± 1.4	19.40 ± 2.6	18.80 ± 3.0
4	17.32 ± 1.8	18.30 ± 1.6	18.80 ± 1.2	19.20 ± 1.6
5	20.22 ± 1.9	20.24 ± 2.0	20.20 ± 2.8	22.80 ± 1.8
6	19.48 ± 2.5	20.44 ± 1.2	18.80 ± 1.6	19. 84 ± 2.6
7	22.10 ± 1.0	24.18 ± 2.8	21.98 ± 2.4	22.40 ± 1.6
8	20.30 ± 1.8	24.24 ± 3.2	21.66 ± 2.8	21.22 ± 1.2
9	18.00 ± 1.2	20.00 ± 2.0	18.74 ± 1.4	19.64 ± 1.9
10	18.08 ± 2.4	21.56 ± 1.8	19. 44 ± 2.2	20.00 ± 2.0

The results presented above show that all isolates were susceptible to the three antibiotics and *Cannabis* extracts in the assay. The methanolic extracts of cannabis exerted pronounced antibacterial activity and were effective in inhibiting the growth of all isolates (18.80-22.80 mm), with the highest zone of inhibition recorded against isolate 5 (22.80 ± 1.8), outperforming all antibiotics tested. The result obtained corresponds with the findings of Ali *et al.* (2012) and Novak *et al. (*2001) who showed that extracts of *Cannabis sativa* have comparable antimicrobial activity due to the presence of sesquiterpenes or cannabinoids. The results obtained in this study shows that methanol extract of cannabis was able to inhibit the test organisms significantly more than the antibiotic chloramphenicol which has been reported to be losing its efficacy in the control of some pathogens (Das and Patra, 2017). The β-lactam antibiotics (Penicillin G and Amoxicillin) showed the greatest activity against the test organisms, recording the highest zones of inhibition. This result is in agreement with the findings of Camara *et al. (*2013) who studied the antibiotic susceptibility pattern of *Streptococcus pyogenes* isolated from respiratory tract infections in Dakar, Senegal. The results obtained from this study is encouraging to note that commercial antibiotics currently in use to combat strep throat. However, the mid-level susceptibility of the isolates to chloramphenicol indicates that although the drug can effectively be used in the combat of the pathogens, there is a high risk for the development of drug resistance. This can lead to infections of strep throat by multidrug-resistant pathogens which is of global concern because of the difficulties in the treatment of such infections (Raloff, 1998).

3.4. Measurement of Minimum Inhibitory and Bactericidal Concentration (MIC and MBC)

Tube dilution assay to measure the inhibitory effect of reducing concentrations of the *Cannabis sativa* extract as presented in Table 3. below shows that 20 mg / ml of the extract was able to inhibit the growth / multiplication of the pathogen with no change in the turbidity of the culture media indicating that bacterial growth did not occur. However, this concentration of the extract was not able to completely kill the organism as upon subculture onto fresh solid media without the antimicrobial extract, growth was recorded by inoculums transferred from tubes containing lower concentrations of the extracts. The complete killing of the bacterial cells was achieved by a higher concentration (30 mg / ml) in which no growth occurred upon subculture, indicating a bactericidal effect.

The accurate and timely identification and treatment of Lancefield Group A *Streptococci* (GAS) is of paramount importance because of the possibility of a relatively mild skin or throat infection to progress rapidly to a life-threatening invasive condition (Langlois and Andreae, 2011; and Lynskey *et al.*, 2011) which can be complicated by resistance to commonly utilized antibiotics. The MBC of methanol extracts of *Cannabis sativa* in this study was recorded as 30 mg / ml. This concentration is comparable to that of the antibiotics currently in use commercially, indicating the potential of extracts of the bioactive ingredients of cannabis to be

Table 3: The minimum inhibitory and bactericidal concentration of extracts

	Concentration (mg/ml)	Turbidity produced (MIC)	Growth on subculture (MBC)
Methanol extract	30	Clear	No growth
	25	Clear	Growth
	20	Clear	Growth
	15	Turbid (+)	-
	10	Turbid (++)	-
		MIC = 20 mg/ml	MBC = 30 mg/ml

used as an antimicrobial agent. This is evident by the practices of traditional healers who underpin many treatments of numerous human ailments with infusions of cannabis extracts (Begum and Nath, 2000).

It is important to unravel alternate approaches for elimination of pathogens from the human body because of the increasing resistance of microorganisms to commercially available antibiotics. Thus, natural phytochemicals from plants have shown potential as alternative antimicrobials, with the plants serving as a common source of medication either in its natural state or in the form of traditional preparation or as commercialized drugs after the extraction of the active components (Asati *et al.*, 2017). Lancefield Group A *Streptococcus* are important pathogens not only with regards to the causation of debilitating skin, oral and systemic infections in healthy and immune-compromised individuals, but they have also been shown to be increasingly resistant to conventional antibiotics available for use. This rise in antibiotics resistance may be attributed to the wrong prescription of antibiotics in all cases of pharyngitis without a laboratory diagnosis of *Streptococcus* sp. even in cases of pharyngitis caused by viruses. It is reported that acute pharyngitis is one of the most misdiagnosed ailments that leads to the inappropriate use of antibiotics in clinical practice (Nakhoul and Hickner, 2013). Thus, to prevent the spread of resistance and encourage rapid elimination of the pathogen, a combination therapy approach can be employed to control the organisms using normal antibiotics in conjunction with plant extracts such as those of cannabis which has been shown to be effective against *Streptococcus* sp. or by the use of purified bioactive components of the herb as practised by alternative medical practitioners.

4. Conclusion

In conclusion, this research has shown the presence of bioactive compounds in *Cannabis sativa* which may be responsible for the antimicrobial activity. Methanolic extracts of the leaves and seed of cannabis is effective in the inhibition and death of Lancefield Group A *Streptococcus* sp. responsible for streptococcal pharyngitis (strep throat). The extracts compared favorably with commercially available β-lactam antibiotics (Penicillin G and Amoxicillin) and performed better than the phenicol chloramphenicol, giving a greater zone of inhibition. It is established that the MIC of cannabis is 20 mg/ml while the MBC is 30 mg/ml. These dosages are low enough to be administered without manifestation of the psychoactive attributes of the herb which has been the limitation in its administration. Further studies need to be undertaken to individually test the properties of the phytochemicals and possibly work towards the formulation of medications using these extracts either for topical, oral or systemic use.

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