Research Article

In vitro antibacterial activity of *Cannabis sativa* (Hemp) extracts against avian pathogenic *Escherichia coli* (APEC) isolated from Broilers Chicken

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Abstract

Objective: To investigate the antibacterial activity of ethyl acetate and methanol extracts of *Cannabis sativa* (*C. sativa*) on avian pathogen. **Material and methods:** Avian Pathogenic *Escherichia coli* (APEC) was isolated from 200 diseased broilers chicken. The heart swab and faecal samples of the naturally infected broilers chicken were collected from poultry farm of College of Agriculture, Jalingo, Taraba State and the associated avian bacteria was isolated and identified through cultural and biochemical methods. The seed and leaves powder were extracted successively with 500 ml of ethyl acetate and methanol solvents by using Soxhlet extractor for 8hr at a temperature not exceeding the boiling point of the solvent. Ethyl acetate and methanol extracts were subjected to phytochemical analysis as well as the antimicrobial assay using agar well diffusion method. **Results and conclusion:** The yields of the extracts were in the order; ELE (48.5%) > MLE (28%) > MSE (14.4%) > ESE (7.8%). Ethyl-acetate seed and leave extracts showed the highest antibacterial activity of 21.50±0.71mm and 21.00±1.41mm respectively against *E. coli* at 2000µg/ml. The findings also revealed that the antibacterial activities of *C. sativa* plant extracts were in the following order; ESE>ELE>MSE>MLE. The present study revealed that the extracts *C. sativa* seed and leaves possess significant antibacterial activity on isolated avian pathogen. To conclude the present findings, the ethyl acetate seed and leaf extracts could be used as potential sources of antibacterial agents.

Keywords: Avian, pathogen, poultry, *Cannabis sativa*, phytochemical, antibacterial

Introduction

Plants are pivotal to the existence of life and plays crucial role in health and nutrition. The use of plant parts for therapeutic or curative purposes cannot be over-emphasized as they containessential bioactive compounds used for myriad of pharmacological purposes (Sofowora et al., 2013; Maria, 2016). There exists in literatures considerable report in recent times on research work on the use of medicinal plants and their constituents in disease prevention as plant parts have attained commanding role in health system all over the world (Sandberg and Corrigan, 2001; Oladeji, 2016). Some of the plants have been found to possess significant antibacterial, antifungal, anticancer, antidiuretic, anti-inflammatory and anti-diabetic properties (Sule et al., 2010; Timothy et al., 2012; Adelowo and Oladeji, 2016). Whole plants, leaves and wood have environmental uses, bark, fiber and seeds are also of ritual importance (Esra et al., 2012; Abubakar et al., 2020).

The introduction of plant derived drugs in modern medicine has been linked to the uses of plant derived materials as an indigenous cure in traditional system of medicine (Igoli et al., 2003; Moghadamtousi et al., 2015). *Cannabis* plant contains a classical cannabinoids, a unique group of secondary metabolites found in the cannabis plant, which are responsible for the plant's peculiar pharmacological effects (Fischer et al., 2015). In medicine, cannabis is grown, processed and used to ease pain, sooth influence in nervous disorders, management and treatment of gout, neuralgia, rheumatism, insanity, insomnia, etc (Mohammed et al., 2013; Ayenigbara, 2014).

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Pathogenic bacteria are involved in causing serious infectious diseases that successively result in mortality and morbidity among the human population, with animals inclusive, especially in the developing countries (Muhammad et al., 2018). The pharmaceutical industries are keen to developing new drugs due to the constant emergence of microbial resistance to conventional medicines (Zakeri and Kashefi, 2012). For example, the multi-drug resistance of Avian Pathogenic *Escherichia coli* (APEC) has been placed among the most stubborn pathogenic bacteria against antibiotic efficacy (Kunert et al., 2015; Xu et al., 2018). This bacteria strain is responsible for a large number of extra-intestinal diseases in birds, either locally or as systemic infections and are responsible for economic losses for the world's poultry industries (Ma et al., 2012; Mariana et al., 2018). Due to frequent outbreaks of viral and bacterial diseases – possibly as a result of the prevalence of antibiotic resistance genes acquired by these organisms at the course of time, poultry industries often suffer a great deal of setbacks (Namdeo et al., 2015; Chandrahas et al., 2017). Hence, the urgent need of this research to determine the antibacterial activities of the extracts of *Cannabis sativa* against Avian Pathogenic *Escherichia coli* (APEC).

Materials and Methods

Study Area

The study was carried out at the Department of Animal Health Laboratory, College of Agriculture, Jalingo, Taraba State. In addition, the antibacterial activity test was carried out in the Department of Microbiology, Bayero University Kano, while the plant extraction and phytochemical screening was conducted at the Department of Chemistry, Bayero University Kano.

Ethical approval and collection of plant sample

The ethical approval was obtained from National Head Quarter, National Drug Law Enforcement Agency (NDLEA) Abuja, Nigeria. One kilogram (1kg) of dried leaf and seed of *Cannabis sativa* (Indian hemp) were obtained from Taraba State Command, National Drug Law Enforcement Agency (NDLEA), Jalingo, Taraba State.

Processing and extraction of plant material

Cannabis sativa (Indian hemp) were confirmed at the Department of Plant Biology, Bayero University Kano. The samples were grounded into powder using mortar and pestle as described by Mukhtar and Tukur (1999). The extraction of the plant material was conducted using Soxhletapparatus (Pyrax Company, United Kingdom). Ethyl-acetate and methanol solvents were used for the extraction (Redfern et al., 2014).

One hundred and eighty grams (180g) of the powdered plant leaf and seed materials were filled in cotton sacked material and introduced inside the Soxhlet extractor. Subsequently, 500ml of ethyl-acetate was also introduced into the soxhlet extractor. The side arm was lagged with glass wool and the solvent was heated using hotplate, the condenser on the isomantle beginning to evaporate moving through the apparatus to condenser, the condenser thin dripped into the reservoir containing the plant materials. As the level of solvent reached the siphon, it poured back into the flask and the circle continued until the plant material colour turned to colourless, and the extracted material poured into the beaker. The process was done for each of the solvents, with the same gram of the plant material. The extracts were left to air dry for the solvent to evaporate and the fraction was later obtained.

Phytochemical Tests

The phytochemical analysis of ethyl acetate and methanolic seed and leaf extracts was carried to find the presence or absence of active secondary metabolites such as alkaloids, tannins, flavonoids, saponin, glycoside, phenols and steroid/triterpenoid by adopting standard protocols of Sofowora (1982) and Trease and Evans (1983). Exactly 0.1g of each of leaf and seed extract of *Cannabis sativa* was separately dissolved into 10ml of ethyl acetate and methanol solvents which were introduced into the beaker which served as a working solution (Edeoga et al., 2005).

Isolation of avian pathogenic *Escherichia coli* (APEC) from chicken

Heart swab and faecal samples were collected from 200 sick broilers chicken in poultry farm, College of Agriculture, Jalingo, Taraba State and was introduced in a sterile polythene bag for isolation/culturing. The samples were cultured in Eosin Methylene Blue (EMB) agar as described by Cown (1974) and incubated for 24hrs at 37°C. The groose colony morphology was observed after 24hrs incubation and the result was recorded.

Identification of the Isolates

The bacterial isolate was identified by observing growth morphology on culture media, Gram staining properties and motility as described by Merchant and Packer (1997). The biochemical tests of the bacterial isolate was carried out according to the method described by Cheesbrough (2006).

Maintenance of Stock Culture

Nutrient agar slant was used to maintain the stock culture for APEC isolate. The isolate was inoculated in a slant by streaking and was incubated at 37°C for 24hrs and the glycerol was added and the isolate was kept at room temperature (CLIS, 2015).

Determination of antibacterial activity of cannabis extracts

Preparation of MacFarland Standard

This was prepared according to the method described by Cheesbrough (2000). A 1.0ml of H_2SO_4 was added to 99ml sterilized distilled deionised water to arrive at 1% (v/v) solution of the acid. Then 0.5 gram of barium chloride ($BaCl_2.2H_2O$) were dissolve in a 50ml of distilled water, resulting in 1% (v/v) H_2SO_4. Two (2ml) of the turbid solution was transferred to a test tube covered in a well-sealed container in the dark place. The turbidity of the inoculum and that of the MacFarland standard turbidity solution were compared by observing the same quantity of the two in separate test tubes on a white printed paper. This was done by adding a little normal saline until the turbidity of the inoculum was matched with that of the MacFarland Standard (Abdulrashid et al*.,* 2018).

Standardization of Inoculum

The isolates were subcultured onto sterile nutrient agar plates incubated at 37°C for 24 hours. Using a sterilized wire loop, the overnight cultures were diluted in normal saline (0.85% w/v) such that their turbidity matches with 0.5 Macfarland standards which give a mean of 1.0×10^8cfu/ml microbial population density (Abdulrashid et al*.,* 2018).

Antibacterial Assay of Fractionated Extracts

The antibacterial assay was done using agar well diffusion method as described by Nester *at al.* (2004) was followed. Mueller Hinton agar was prepared as specified by the manufacturer; the media was autoclaved at 121°C for 15minutes and poured aseptically into sterile petri-dished and allowed to gel. A loopful of standardized isolate suspension was streaked evenly on each agar plate.Stock solution of (4000 µg/ml) of leaf and seed fractionated extract of *Cannabis sativa* was separately prepared by dissolving 0.004g of the extract into 1ml of dimethyl sulphoxide (DMSO) to obtain the concentration of 4000µg/ml. From this, the working concentrations of 2000µg/ml, 1000µg/ml and 500µg/ml were made. Then 0.1ml of each fractionated extract was inoculated into three wells (6mm diameter) bore with a sterile cork borer to each plate.A commercial oxytetracycline HCl of 0.03µg/ml was prepared and inoculated into fourth (4^{th}) well to serve as positive control. The plates were then allowed to stand for 30mins on the table for pre-diffusion of the extract and were inoculated at 37°C for 24hrs. The antibacterial activity of the extract was determined after incubation by measuring the mean diameter zones of inhibition produced by each of the extracts against the bacterial species and result was recorded in millimeter (mm).

Determination of Minimum Inhibitory Concentration (MIC)

Broth dilution method was used as described by Clinical Laboratory Standard Institute (CLSI, 2015). The lowest concentration of the extract that inhibits the growth of the test organisms (bacteriostatic) was determined. To each concentration of the extracts i.e. 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml and 31.25µg/ml, a loop full of culture adjusted to 0.5 MacFarland standard was inoculated into it and all the tubes were incubated at 37°C for 24 hours. The tube containing broth and extract serve as the positive control while the tube containing broth and inoculum serves as negative control. The lowest concentration of the extract that inhibited the growth of each organism was considered as minimum inhibitory concentration (MIC).

Statistical Analysis

The results obtained after bioassay were subjected to analysis of Variance (ANOVA). Data for the screening of activity of *Cannabis sativa* extracts against Avian Pathogenic *Escherichia coli* (APEC) was determined by the analysis of variance using SPSS (version 16) and GraphPad Instat 3.0 statistical softwares.

Results

Physical Properties and Phytochemical Constituents of *C. sativa* plant extracts

Cannabis sativa extracts were tested for their physical properties (weight, percentage yield, physical appearance before and after evaporation) as showed in Table 1. The result revealed that the weight yield of the extracts ranged from 14.1g to 87.3g. In order of weight yield, the extracts were ethyl-acetate leave extract (87.3g), methanol leave extract (50.4g), methanol seed extract (26.9g), and ethyl-acetate seed extract (14.1g). There were changes in the consistency of the plant extracts after the evaporation of extracts as compared to the appearance of the samples before the evaporation of extracts.

Phytochemical components were screened in the extracts of *C. sativa.* The result of the phytochemical assay of *C. sativa* extracts revealed that in methanol seed extract (MSE), only two (tannins and triterpenoids) of the eight screened bioactive compounds were found (Table 2). Methanol leave extract (MLE) was observed to have more bioactive contents than other extracts of the same plant. The phytochemicals constituents of ethyl-acetate seed extract (ESE) were tannins, steroids, saponins and triterpenoid. The result also showed that tannins, steroids, glycoside and triterpenoid were observed in ethyl-acetate leave extract (ELE) (Table 2).

Cultural and biochemical characterization of bacterial isolates from Chicken

The result of the cultural and biochemical characteristics of

Table 1. Physical properties, weight and percentage of *C. sativa* plant extracts

Plant Extracts	Physical Appearance		Weight (g)	Percentage Yield (%)
	Before evaporation of extract	After evaporation of extract		
ESE	Yellowish oily liquid	Yellowish oily solid	14.1	7.8
ELE	Dark green oily liquid	Dark green gummy solid	87.3	48.5
MSE	Brownish oily liquid	Brownish oily solid	26.9	14.4
MLE	Green liquid	Dark green gummy solid	50.4	28.0

Keys: ESE = ethyl-acetate seed extract, ELE = ethyl-acetate leave extract, MSE = methanol seed extract, MLE = methanol leave extract.

Table 2. Phytochemical constituents on *C. sativa* plant extracts

Plant Extracts	Phytochemical components							
	Alkaloid	Flavonoid	Tannins	Phenol	Steroids	Glycoside	Saponins	Triterpenoid
ESE	−	−	+	−	+	−	+	+
MSE	−	−	+	−	−	−	−	+
ELE	−	−	+	−	+	+	−	+
MLE	+	+	+	+	−	+	+	−

Keys: − = Negative, + = Positive, ESE = ethyl-acetate seed extract, ELE = ethyl-acetate leave extract, MSE = methanol seed extract, MLE = methanol leave extract.

the test organisms indicated that *E. coli* was a Gram negative, rod-like and motile bacterium and reacted positive to Methyl red, indole and Triple Sugar Iron tests. Also the bacterial isolate reacted negative to catalase, coagulase, oxidase, Vogues Proskauer, citrate, urease and hydrogen sulphide (Table 3).

Antibacterial activity of *C. sativa* extracts on bacterial isolate

The antibacterial activities of *Cannabis sativa* extracts against *Escherichia coli* ranged from 7.50±0.71 to 21.50±0.71mm. Ethyl-acetate seed and leave extracts showed the highest antibacterial activity against *E. coli* as ethyl-acetate seed extract (ESE) inhibited bacterial growth at 2000µg/ml (zone of inhibition of 21.50±0.71mm). Ethyl-acetate leave extracts (ELE) had the next highest antibacterial activity at 2000µg/ml (zone of inhibition of 21.00±1.41mm). The result also showed that the antibacterial activities of *Escherichia coli* plant extracts were in the following order, ESE>ELE>MSE>MLE. Similar to the effect of methanolic seed and leave extracts to *C. sativa*, methanolic seed and leave extracts were observed to exact the least bacterial activities (zone of inhibition of 7.50±0.1mm) respectively (Table 4).

Minimum Inhibitory Concentration (MIC) of *C. Sativa* plant extracts

The MIC range of 62.50 to 125µg/ml of *C. sativa* plant extracts was observed to inhibit the activity of *Escherichia coli*. The result also showed that the MIC of ethyl-acetate leave extract against *Escherichia coli* was the highest (125µg/ml) while MIC of 62.50µg/ml on *Escherichia coli* was observed for ethyl-acetate seed, methanol seed and leave extracts (Table 5).

Discussion

The phytochemical content of *Cannabis sativa* gives the plant

Table 3. Cultural and Biochemical characterization of the bacteria isolates

Microscopic Morphology	Gram stain	Catalase	Coagulase	Oxidase	MR	V-P	Indole	Citrate	Urease	Motility	TSI	H_2S	Identified Bacteria
Rod	–	–	–	–	+	–	+	–	–	+	+	–	*Escherichia coli*

Keys: + = Positive, – = Negative, TSI = Triple Sugar Iron, MR = Methyl red, VP = Vogues Proskauer, H_2S = Hydrogen sulphide

Table 4. Antibacterial activity of *C. sativa* plant extracts against *Escherichia coli*

Plant extracts	Concentration (µg/ml) / Zone of Inhibition (mm)			
	2000	**1000**	**500**	**Control (+ve) (0.03µg)**
ESE	21.50±0.71	20.50±0.71	18.50±0.71	23.10±1.51
ELE	21.00±1.41	19.50±0.71	9.50±0.71	30.00±1.55
MSE	10.50±0.71	9.50±0.71	7.50±0.71	23.00±1.51
MLE	9.00±0.00	7.50±0.71	7.50±0.71	22.00±0.75

Keys: ESE = ethyl-acetate seed extract, ELE = ethyl-acetate leave extract, MSE = methanol seed extract, MLE = methanol leave extracts. +ve = Positive, –ve = Negative. Values are means ± SD of two replicates.

Table 5. MIC of *C. sativa* plant extracts on bacterial isolate

Plant extracts	Concentration (µg/ml) (MIC)
ESE	62.50
ELE	125.00
MSE	62.50
MLE	62.50

Keys: ESE = ethyl-acetate seed extract, ELE = ethyl-acetate leave extract, MSE = methanolic seed extract, MLE = methanol leave extracts.

myriad of pharmacological potentials which may result in beneficial end use or may cause detrimental effect to health. The findings on the physicochemical constituents of *C. sativa* indicated that the yield of extract recovered varies with the type of solvent and the plant part used. The ethyl-acetate leave extract produced a higher yield (48.5%), followed by methanol leave extract (28%), while ethyl-acetate seed yielded the least amount of extract (7.8%). The yields of the extracts were in the order ELE>MLE>MSE>ESE. Ncube et al. (2008) reported that, amount of yield produced depend on the solvent used and variations in extraction methods as observed in the present study.

The phytochemical analysis conducted on *C. sativa* leaves and seeds extracts revealed the presence of bioactive compounds such as alkaloid, flavonoid, tannins, phenol, steroids, glycoside, saponins and triterpenoid. These compounds corresponded with those reported by Aslam et al. (2009). Similarly, Abubakar et al. (2020) in their studies on the phytochemical constituents of petroleum ether, ethyl-acetate and methanolic extracts of *C. sativa* leaves and seeds confirmed that variations in extraction methods resulted to some variation in the identified bioactive compounds. This is as the methanolic extract of the leaves contain more phytochemicals than those found in ethyl-acetate plant extracts. This result correspond with the report of Nataranjan et al. (2003) who reported that different plant extracts give rise to varying amount of phytochemicals. Furthermore, variation between the various extracts in phytochemical constituents caused by the extraction ability of a particular component which appear to depend on extraction medium (solvent) polarity and the ratio of solute to solvent as well as increase in temperature (Simon et al., 2015). The presence of these secondary metabolites in plants produces some biological activity in man and animal and is responsible for their use as herbs. These compounds also serve to protect the plant against infection by microorganisms, predation by insect and herbivores while some give plants their characteristic odors and flavors. Some still are responsible for their pigments (Patel, 2010). In some cases, the activity has been associated with specific compounds or classes of compounds. These active constituent can be used to search for bioactive lead compounds that could be used as precursors for the synthesis of more useful drugs (Ogbonnia et al., 2008; Abubakar et al., 2020).

Avian pathogenic *Escherichia coli* (APEC) was isolated and identified through standard cultural and biochemical techniques. Cultural morphology of APEC on Eosine Metheylene Blue (EMB) was in accordance with the findings of Park et al. (2012) and Ferdous et al. (2013). Differentiation of *E. coli* done by specific sugar fermentation tests concurs with the report of Rahman et al. (2014). Biochemical reactions reported during present investigation were classical findings of APEC (Mir et al., 2015; Dey et al., 2016). The findings on the antibacterial activities of *C. sativa* plant extracts against *Escherichia coli* showed the range of 7.50±0.71 and 21.50±0.71mm. Ethyl-acetate seed and leave extracts showed the highest antibacterial activity of 21.50±0.71mm and 21.00±1.41mm respectively against *E. coli* at 2000µg/ml. The result also showed that the antibacterial activities of *C. sativa* plant extracts were in the following order;

ESE>ELE>MSE>MLE. All plant extracts inhibited the activities of the test organisms at varying concentration. Considering the bioactive contents of *C. sativa*, as previously reported by Kang et al. (2010), the extracts can serve potent and effective therapeutic use. This antibacterial potential of *C. sativa* leaves and seed extracts may be due to it constituents. Indeed, the phyto-constituents alkaloids, glycosides, flavanoids and saponins which are importance components of *C. sativa* contain antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens (Orhue et al*.,* 2014).

Furthermore, the susceptibility test of *E. coli* to the plant isolates is an indication that the isolates can be harnessed as alternative treatment remedy for bacterial avian infection (Marano et al*.*, 2000; Clemente et al*.*, 2013; Liebana et al*.,* 2013; Ferreira et al*.,* 2014). The minimum inhibitory concentrations (MIC) range of 62.50µg/ml to 125µg/ml of *C. sativa* plant extracts was observed to inhibit the activity of *Escherichia coli*. The result also showed that the MICs of ethyl-acetate leave extracts against *Escherichia coli* were the highest at 125µg/ml.

Conclusion

The present study has revealed that ethyl-acetate and methanol seed and leave extracts had significant antibacterial effect against *Escherichia coli*. The yields of the extracts were in the order; ELE (48.5%) > MLE (28%) > MSE (14.4%) > ESE (7.8%). Ethyl-acetate seed and leave extracts showed the highest antibacterial activity of 21.50±0.71mm and 21.00±1.41mm respectively against *E. coli* at 2000µg/ml. The result also showed that the antibacterial activities of *C. sativa* plant extracts were in the order; ESE>ELE>MSE>MLE. Thus, *Cannabis sativa* plant extracts are viable therapeutic agents for the treatment of avian diseases. Although extracts from various parts of *Cannabis sativa* have medicinal applications from time immemorial, very little work has been done on the biological activity and plausible medicinal applications of isolated compounds. Hence drug-development programmes could be undertaken to investigate the bioactivity, mechanism of action, pharmacokinetics and toxicity of compounds isolated from *Cannabis sativa* plant.

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