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PHYTOCHEMICAL AND ANTIBACTERIAL SCREENING OF LEAF EXTRACTS FROM *Cassia singueana* Del. (Fabaceae)

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ABSTRACT

Antibacterial resistance is on the rise and new antibacterial drugs are urgently required. Medicinal plants constitute a great source for new antibacterial drugs. Cassia singueana has been used as folk remedy for the treatment of gonorrhoea, bilharzia, constipation, sterility in women, urinary and respiratory tract infections. However, only one study have reported the antibacterial activity of leaf extracts of C. singueana. Herein, the phytochemical and antibacterial activity of n-hexane, Ethyl acetate and Methanol leaf extracts of C. singueana is reported. The antibacterial activity of the extracts were evaluated using the tetrazolium microplate assay in 96-well microplates.Phenol was detected in all the extracts. Flavonoid, tannins, triterpenoids, phytosterol, saponin and anthraquinones were present in the Ethyl acetate extract while glycoside was detected in the Methanol extract. The Methanol extract showed broad-spectrum activity against all the tested bacteria with a promising antibacterial activity against Bacillus subtilis15.6µg/mL.Moderate activity in the range of 125 to 250 µg/mL (both MIC and MBC) was displayed by the Ethyl acetate extracts against Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, and Pseudomonas aeruginosa. The high and medium polar extracts of the leaf of C. singueana was observed to contain phytochemicals with promising activities against both Gram-negative and Gram-positive bacteria.

Keywords: Antibacterial, Cassia singueana, flavonoid, Fabaceae, Minimum inhibitory concentration

INTRODUCTION

Infections due to antimicrobial resistance has continue to be a major medical problem and threat to human health. It has been projected that the problem of antimicrobial resistance will cause an estimate of 50 million deaths globally by 2050 (Manuka et al., 2017).The situation of antibiotic-resistant is most particular for bacterial such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Proteus spp.* (Claudia et al., 2017; Manuka et al., 2017).The search for novel antimicrobial drug is quite challenging and lengthy process (Nur et al., 2016). Thus, plant derived substance as alternative to lead compound source is required to help fight the deadly infections.

Cassia singueana (Fabaceae), commonly known as winter cassia, is a shrub widely distributed across India and tropical Africa including Nigeria (Schmelzer and Gurib-Fakim, 2008). The plant is used in the treatment of gonorrhoea, bilharzia, constipation, stomach-ache, diabetes mellitus, sterility in women, urinary schistosomiases, hernia, ulcer, malaria, heartburn,snake bite and respiratory tract infections (Bulus et al., 2003; Ode et al., 2012; Bilkisu et al., 2019).The antiplasmodial activities of the root extract (Bulus et al., 2003),*in vitro* anti-histamine (Ode et al., 2012)and the *in vivo* anti-ulcer activity of the leaf (Ode, 2011) has been reported. Only one study reported the antibacterial effect of the ethanol and aqueous extracts from the leaf of *C. singueana* (Olusola et al., 2011). This study report the phytochemicals and antibacterial activity of various extracts obtained by solvent of different polarity from the leaf of *C. singueana*.

MATERIALS AND METHODS

Plant Material

The leafof *C. singueana* was collected in August, 2014, from Kumuyel, Alkaleri Local Government Area of Bauchi State, Nigeria. The plant was identified by Mr. Baha'uddeen Said
Adam, a voucher specimen, (BUKHAN 0316) was preserved at the Herbarium of Department of Plant Biology, Bayero University Kano, Nigeria.

Plant Extraction

The air-dried and powdered leaf of *C. singueana* (200 g) was extracted successfully in *n*-hexane, ethyl acetate and methanol (each 3 × 4 L) at room temperature. The extract was filtered and concentrated under reduced pressure to yield the *n*- hexane leaf extract (CSLH, 3.2 g, 1.6%), the ethyl acetate leaf extract (CSLE, 5.4 g, 2.7%) and the methanol leaf extract (CSLM, 6.8 g, 3.4%) respectively. The extracts were screened for their phytochemical and antibacterial activity.

Phytochemical screening

Qualitative phytochemical screening of leaf extracts from *C. singueana* was conducted using standard methods (Nahar&Satyaji, 2007; Bandiola, 2018).

Flavonoids

Shinoda's test

To an aliquot (1 mL) of the extracts, hydrochloric acid (0.5 mL) and magnesium metal was added. A reddish coloration indicate the presence of flavonoid.

Alkaline reagent test

Few drops of sodium hydroxide solution was added to 1 mL of each extracts. The formation of an intense yellow colour, which changes to colourless on addition of dilute hydrochloric acid, shows the presence of flavonoids.

Tannins

Ferric chloride test

A few drops of 5% ferric chloride solution was added to 1 mL of each extract. The appearance of dark green colour signifies the presence of tannins.

Triterpenoids

Salkowski's test

Test extract wasmixed with chloroform and then filtered. Then few drops of conc. sulphuric acid was added and shaken before allowed to stand. The formation of red brown or golden yellow colour shows the presence of triterpenes.

Phytosterols

Libermann-Burchard's test

Test extract was mixed with chloroform and then filtered. The filtrate was treated with a few drops of acetic anhydride, boiled, and cooled. Then conc. sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

Saponins

Foam test

Solution of each extract was shaken vigorously for 10 minutes. Frothing which last for about 10 minutes indicates the presence of saponins.

Alkaloids

Wagner's test

Few drops of Wagner's reagent was added to a 2 mL of each extract. A reddish- Brown precipitate indicates the presence of alkaloids.

Hager's test

Few drops of Hager's reagent was added to acidified solution of each extract. Precipitates appear as positive test.

Mayer's test

Two drops of Mayer's reagent was added to acidified solution of each extract along the sides of test tube. Formation of precipitate indicates the presence of alkaloids.

Dragendroff's test

Few drops of Dragendroff's reagent was added to acidified solution of each extract. The presence of alkaloids is indicated by formation of red precipitate.

Glycosides

Few drops of dilute hydrochloric acid was added to aliquot of each extract and warm for 30 minutes on a water bath. Fehling's solution A and B was then added and warm gently.A brick red colouration indicates a positive test.

Phenols

Lead acetate test

10% lead acetate solution (2 mL) was added to aliquot of each extract. Formation of white precipitate indicates the presence of phenolic compounds.

Anthraquinones

0.2 g of each extract was boiled with 10 ml ofsulphuric acid (H_2SO_4) and filtered. The filtrate was shaken with 5 ml ofchloroform then 1 ml of dilute ammonia solution was added. A pink or violet colour in the base layer indicates the presence of anthraquinones.

Bacterial strains

Three Gram-positive bacteria; *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC29737), *Enterococcus faecalis* (ATCC19433), and three Gram-negative bacteria; *Pseudomonas aeruginosa* (ATCC9027), *Escherichia coli* (ATCC10536), *Klebsiella pneumoniae* (ATCC13883) were used as test bacteria. All the bacteria were grown in Nutrient Broth at37°C and maintained in Nutrient Agarat 4°C.

Preparation of crude extracts and antibiotics

The plant extracts were dissolved in 50 % dimethylsulfoxide (DMSO) in sterile Mueller Hinton broth to obtain working concentration of 2 mg/ml. The final concentration of DMSO in the well was ensured to be less than 2 %. Streptomycin were prepared to a final concentration of 0.1 mg/ml which served as positive drug control against the bacterial strains.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination

The MIC evaluations were performed in triplicates using tetrazolium microplate assay as described by Eloff (1998) with slight modifications. This assay was performed using 96-well plates. The wells in column A of each row were left blank and the last seven wells from column B to H were filled with 100 µl of sterilized Mueller Hinton broth. Working solution of plant extracts were added to the wells in column A and B of each row and an identical two-fold serial dilution were made from column B to the column G. The last wells in column H was served as drug-free controls. An appropriate solvent blanks(DMSO) were included as negative control. Finally, 100 µl of bacterial inoculum were added in all the wells from column A to H and mixed thoroughly to give final concentrations ranging from 1000µg/ml –15.625 µg/ml. The cultured microplates were incubated at 37 °C for 24 h. The MIC of samples was detected following addition (50 µl) of 0.2 mg/ml p-iodonitrotetrazolium chloride (which serves as indicator) in all the wells and incubated for further 30 min at 37°C. Bacterial growth was determined by observing the colour change of p-iodonitrotetrazolium chloride in the microplate wells (reddish-pink colour when there is growth and clear solution when there is no growth). MIC was defined as the lowest sample concentration showing nocolour change (clear) and exhibited complete inhibition of bacterial growth. Microorganism with MIC values higher than 500µg/ml were regarded as not active against the tested plant extracts.

For the determination of minimum bactericidal concentration (MBC), aliquot of liquid from each well that showed no change in colour was placed on Mueller Hinton Agar and incubated at 37^{o}C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC.

RESULTS

Phytochemical analysis

The phytochemical study of leaf extracts of *C. Singueana* (**Table 1**) revealed the presence of phenols in all the tested extracts. Alkaloids was not detected in any of the extracts. Only the ethyl acetate extract showed the presence of flavonoid. However, triterpenoid, phytosterol and anthraquinones were not detected in the methanol leaf extract of *C. singueana*.

Table 1: Phytochemical constituents of leaf extracts of *C. singueana*

Tests	CSLH	CSLE	CSLM
Flavonoid	-	+	-
Tannins	-	+	+
Triterpenoids	+	+	-
Phytosterol	+	+	-
Saponin	-	+	-
Alkaloids	-	-	-
Glycosides	-	-	+
Phenols	+	+	+
Anthraquinones	+	+	-

Present: +; Absent: -

Antibacterial Assay

The results for antibacterial screening of leaf extracts of *C. singueana* are shown in **Table 2**. The determination of the leaf extracts against selected bacterial strains recorded a minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values in the range of 15.6 – 1000 µg/ml. (**Table 2**). MIC values lower than 100 µg/mL are considered promising activity; between 100 and 300 µg/mL denotes moderate activity; 300 – 500 µg/mL correspond to weak activity, while above 500 µg/mL represent inactivity (Gibbson, 2004; Rios and Recio, 2005). The methanol leaf extract of *C. singueana* demonstrated broad-spectrum activity against all the tested bacteria with a promising activity (15.6 µg/mL) against *Bacillus subtilis*. Moderate activity (125 – 250 µg/mL) was observed for the ethyl acetate extract against *S. aureus*, *Bacillus subtilis*, *Enterococcus faecalis* and *P. aeroginosa*, while both *n*-hexane and ethyl acetate extract displayed weak activity (500µg/mL) against *Klebsiella pneumoniae*. However, the *n*-hexane extract was inactive (1000 µg/mL) against *E. coli* and *P. aeruginosa.*

Table 2: Minimum Inhibition Concentration (MIC) and Minimum bactericidal Concentration (MBC) of leaf extracts of *C. singueana*

Sample	Test	**Microorganisms**					
		Gram positive bacteria			**Gram negative bacteria**		
		SA	**BS**	**EF**	**EC**	**KP**	**PA**
CSLH	MIC	500	500	250	1000	500	1000
	MBC	500	500	125	ND	500	ND
CSLE	MIC	250	125	250	500	500	125
	MBC	125	125	250	500	500	125
CSLM	MIC	250	15.6	125	250	250	250
	MBC	250	15.6	62.5	250	250	125
Positive control Streptomycin sulphate	MIC	3.13	1.56	3.13	6.25	6.25	3.13
	MBC	3.13	1.56	3.13	6.25	6.25	3.13

CS = *Cassia singueana*; L = Leaf; H = *n*-hexane; E = ethyl acetate; M = methanol; SA = *Staphyloccocus aureus*; BS = *Bacillus subtilis;*EF = *Enterococcus faecalis*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae;* PA = *Pseudomonas aeroginosa*; MIC = Minimum inhibition concentration (µg/ml); MBC = Minimum bactericidal concentration (µg/ml); ND = not determined

DISCUSSION

The leaf of *C. singueana* was extracted in solvent of increasing polarity; *n*-hexane, ethyl acetate and methanol. However, majority of the secondary metabolites; flavonoid, tannins, triterpenes, phytosterol, saponins, phenols and anthraquinones, were detected in the medium polar extract, (ethyl acetate extract).Study on the leaf of *C. singueana* collected from Blue Nile State, Savanna in Sudan reported the presences of alkaloids, flavonoids, sterol (phytosterol), triterpenes, tannins and glycosides in both ethyl acetate and methanol extracts from the leaf of *C. singueana* (Missa et al., 2015). Similar findings has been reported for ethyl acetate and ethanol leaf extracts from *C. Singueana* (Debes et al., 2018).

These group of secondary metabolites detected in the medium and high polar extracts of leaf of *C. singueana* could be responsible for the significant antibacterial activities observed in the methanol and ethanol extracts respectively. Similar finding has shown broad spectrum activity for ethanol leaf extract of *C. singueana* against both Gram-negative and Gram-positive bacteria (Olusola et al., 2011).Secondary metabolites such as flavonoids, alkaloids, phenols, phytosterol and quinone compounds have been reported as potent antimicrobial agents (Gibbsons, 2004; Saleem et al., 2010).

CONCLUSION

The problem of antibacterial resistance has been as a result of multitude ways of drug resistance developed by bacteria. As such, getting around the problem could be achieved using the chemical diversity of plants as means to the resistance problem. The use of medium to high polar solvents in the extraction of the leaf of *C. singueana* reveals the presence of phytochemicals with promising activity against both Gram-negative and Gram-positive bacteria.

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