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PHYTOCHEMICAL SCREENING, ANTIMICROBIAL PROPERTIES AND ESSENTIAL OIL CONSTITUENTS OF COMBRETUM SORDIDUM EXELL

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ABSTRACT: Plants in Combretaceae family are traditionally reputed to be anthelmintics and antimicrobial agents. Dried aerial parts of *Combretum sordidum* were ground to fine powder and extracted using ethanol. Ethanol extract was partitioned to obtain hexane and ethylacetate extracts successively. These extracts were screened for secondary metabolites using standard methods and evaluated for their antimicrobial potentials against two Gram positive bacteria; *Bacillus subtilis* (ATCC 14579) and *Bacillus cereus* (ATCC 33923); two Gram negative bacteria; *Proteus mirabilis* (ATCC 21784), *Salmonella typhi* (ATCC 25179) and a fungus; *Candida albicans* (NCTC 227) by tube dilution method. Essential oil from the leaves was obtained by hydrodistillation method and analyzed by GC and GC-MS. Preliminary phytochemical screening revealed the presence of alkaloids, saponins, tannins, flavonoids, cardiac glycosides, steroids and anthraquinones in the ethylacetate and ethanol extracts. Both extracts displayed broad spectrum activities against the organisms used with minimum inhibitory concentration (MIC) at 400 µg/mL. Five compounds comprising 99.99% of the total peak area were identified from the volatile oil. The most abundant constituent was 7- octadecenoic acid methyl ester (51.26%) and others were dodecanoic acid (21.12%), hexadecanoic acid (11.58%), tetradecanoic acid (8.94%) and octadecanoic acid (7.09%) methyl esters. These secondary metabolites might be responsible for the antimicrobial activities of *C. sordidum.* The result obtained from this study further justifies the use of this plant as antimicrobial agent by the traditional healers.

INTRODUCTION: Members of the *Combretaceae* family are widely used in African traditional medicine for the treatment of microbial infections [1]. Species from the *Combretum* genus and to a lesser extent *Terminalia* are common and distributed throughout Western and Southern Africa [2]. Records indicated that majority of the traditional healers use these species for treating abdominal disorders, backache, conjunctivitis, venereal disease, hypertension, jaundice, pneumonia, toothache, diabetes, tumours, tuberculosis and yellow fever [1-7].

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Compounds such as combregenin, arjunglucoside, combreglucoside, 5, 4 – dihydroxy – 7 - methoxy flavone; 5,4`-dihydroxy-7,5`-dimethoxyflavone; 5,7,4`-trihydroxyflavone; punicalagin, hydrolysable tannins and imberbic acid were among the constituents isolated from *combretum* species with antimicrobial activities [8-9].

The medicinal usefulness of plants has been the subject of numerous chemical and microbiological studies. Some of the reported phytoconstituents of herbs include terpenoids, alkaloids, tannins, saponins, flavonoids and phenanthrenes [4, 5, 8]. And their uses include antisplasmodic, antiasthmatic, antimicrobial and anti-inflammatory [9]. *Combretum sordidum* is a scandent shrub or a creeper. Its flowers are white and easily recognized by the small red scales on the under-surface of the leaves [10]. The ethnopharmacological and medicinal uses of *C. sordidum* has got little or no scientific record

in the literature. In view of this, we report the preliminary phytochemical screening, antimicrobial properties and essential oil constituents of *C. sordidum* for the first time.

MATERIALS AND METHODS:

Plant Collection and Identification: Fresh aerial parts of *Combretum sordidum* were collected in the Year 2013 from the botanical garden, University University of Ibadan, Ibadan, Nigeria. It was identified and authenticated at the herbarium unit of the department of Botany, University of Ibadan, where a voucher specimen was deposited (UIH-22359).

Preparation, Extraction and Partitioning of Plant Material:

The aerial parts were cut into small pieces, air-dried and ground to a fine powder. Plant material (1335g) was soaked in 10L round bottom flask with 8L of ethanol for 72h. Extract was concentrated using rotary evaporator at 40^0C and crude extract (160g) was obtained. Ethanol extract (100g) was subjected to liquid-liquid partitioning using hexane and ethylacetate to obtain the respective fractions.

Phytochemical analysis of extracts:

The ethanol extract and the fractions obtained were subjected to phytochemical screening. The entire tests were carried out using standard methods [11].

Alkaloids:

Each extract was acidified with 1% HCl and treated with few drops of Mayer`s, Wagner`s and Dragendorff`s reagents separately in different test tubes. A creamy white (Mayer), reddish brown (Wagner) or an orange (Dragendorff) precipitate indicated the presence of alkaloids.

Saponins:

Presence of saponins was detected by adding some amount of distilled water to the extracts. After heating the mixture, the filtrate was collected and shaken. Formation of froth revealed the presence of saponins.

Steroids:

Each extract was dissolved in 2mL of chloroform in a test tube. Concentrated H_2SO_4 (0.2mL) was carefully added to form a lower layer. A red colour produced in the lower chloroform layer showed the presence of steroids.

Flavonoids:

Each extract was dissolved in dilute NaOH. A yellow solution that turns colourless on addition of HCl acid indicates the presence of flavonoids.

Cardiac glycosides:

Each extract was dissolved in 2mL of glacial acetic acid containing a drop of ferric chloride solution. This underplayed with 1mL of concentrated Sulphuric acid. A brown ring at the interface revealed the presence of cardiac glycosides.

Anthraquinones:

The extract was shaken with 4mL of benzene. The mixture was filtered and 2mL of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of pink red or violet colour in ammoniacal solution (lower phase) showed the presence of free anthraquinones.

Tannins:

Each test extract was mixed with water and filtered. A dirty green precipitate or blue-black or blue green precipitate on addition of few drops of 5% ferric chloride to the test extract was taken as an indication of the presence of tannins.

Determination of Antimicrobial Activity:

Microorganisms used were standard strains of two Gram positive bacteria; *Bacillus subtilis* (ATCC 14579) and *Bacillus cereus* (ATCC 33923); two Gram negative; *Proteus mirabilis* (ATCC 21784) and *Salmonella typhi* (ATCC 25179) and a fungus; *Candida albicans* (NCTC 227), these were obtained from Centre for Drug Research Institute (CDRI) Lucknow, India. Standard microbial cultures were prepared by sub-culturing a loopful of each microbe into sterile nutrient broth and incubated for 24 h at 37 ^{o}C for the bacteria and 48 h for the fungus. The suspensions were adjusted to a turbidity of 10^5 colony forming unit (cfu/mL).

Minimum inhibitory concentrations (MICs) were determined using the tube dilution methods [12]. Nutrient broth (2mL) was dispensed into each of

the twelve (12) test tubes followed by an addition of the extract (1mL) in the first tube. From the resulting mixtures, 0.5mL of the solution was removed to make serial dilution to the nineth tube excluding the neutral, negative and positive controls. Thereafter, 0.2mL of each organism was added into the test tubes. Gentamicin (0.3mL) and Ethylacetate:water, 1:1, were used as the positive and negative controls respectively. The nutrient broth with organisms only was used as the neutral control. Lowest concentration that showed no growth was the MIC after 24h incubation at 37^0C.

Extraction of Essential Oil:

Fresh leaves (250g) were subjected to hydrodistillation in a Clevenger-type apparatus for 3h. The essential oil was collected over hexane and stored in a sealed vial under refrigeration prior to analysis.

Analysis of the Essential Oil:

Gas chromatography (GC):

The volatile oil was subjected to GC analysis on Shimadzu mol QP2010 system equipped with an AOCi-20i autosampler. The column used was DB5 (30m×0.25mm, 0.25µm film thickness). Helium was used as the carrier gas at a flow rate of 1mL/min, linear velocity of 362 cm/sec and pressure 56.2 KPa. The oven temperature was set at 60 ^{0}C, hold for 1min to 180 ^{0}C at 10^0C/min, and hold for 3mins, the final temperature was 280 ^{0}C at 12 ^{0}C, and hold for 2mins. Both injector and detector temperatures were fixed at 250 ^{0}C.

Gas chromatography- mass spectrometry (GC-MS):

The GC-MS analysis was performed on Shimadzu model QP2010 system model with split/splitless injector interfaced to a 5975 mass selective detector operated at 70 eV, ion source temperature of 200 ^{0}C with a mass range of m/z 50 -700 at scan rate of 1428 amu/sec. The same temperature programme as for the GC was used. Total analysis time was 24 mins.

Identification of components:

Identification of each individual constituent of the essential oil was achieved based on their retention indices (determined with reference to a homologous series of normal alkane) and by comparison of their mass spectral fragmentation patterns (NIST data/base/chemstation data system) with data previously reported in the literature [13].

RESULTS AND DISCUSSION:

The ethanol crude extract gave percentage yield of 11.80 while the yield of hexane and ethylacetate fractions were 1.60% and 3.98% respectively (**Table 1**).

TABLE 1: PERCENTAGE YIELDS OF EXTRACT AND FRACTIONS OF *C. SORDIDUM*

Extracts	Weight (g)	Yield (%)
Hexane	13.58	1.6
Ethylacetate	33.67	3.98
Methanol	159.83	11.8

Preliminary phytochemical screening of the extract and fractions investigated were summarized in **Table 2.** Saponins, flavonoids, cardiac glycosides, alkaloids, tannins and anthraquinone were present in both ethylacetate and ethanol extracts. These classes of components were reported to exhibit a variety of biological activities including antiviral, antibacterial, anti-inflammatory and analgesic, all of which were relevant to the traditional uses of some species of the genus [14]. The phytochemicals also have strong antimicrobial significance against potential enteric pathogens, the presence of alkaloids in appreciable quantities may be used as antimalarial, analgesics and stimulants while tannins are used in treating wounds, sprains, bruises and in arresting bleeding [15-16].

TABLE 2: PHYTOCHEMICAL ANALYSIS OF THE EXTRACT AND FRACTIONS OF *C. SORDIDUM*

Chemical Constituent	Hexane	Ethylacetate	Ethanol
Saponins	+	+	+
Steroids	-	+	+
Flavonoids	+	+	+
Cardiac Glycosides	+	+	+
Alkaloids	+	+	+
Tannins	+	+	+
Anthraquinones	+	+	+

Keys: + = Present, - = Absent

Antimicrobial activities of the extracts of *C. sordidum* are presented in **Table 3**. The result showed that ethylacetate extract was moderately active against *B. subtilis, S. typhi* and *C. albicans* while ethanol extract was active against *B. subtilis*,

P. mirabilis, S. typhi and *C. albicans,* both extracts with MIC at 400µg/mL. Ethylacetate extract exhibited strong inhibition against *B.cereus* with MIC at 16µg/mL. Hexane extract showed activity against *S. typhi* only with MIC at 400µg/mL. This shows that the antimicrobial constituents are likely to be contained in the ethylacetate and ethanol extracts and also reaffirms the ethnopharmacological uses of this plant as an antimicrobial agent.

The essential oil yield of *C. sordidum* obtained from hydrodistillation of leaves was 0.24%. Five components were identified in the volatile oil accounting for 99.99% of the composition by GC-MS (**Table 4**). These components were fatty acid esters, the most abundant was 7- octadecenoic acid methyl ester (51.26%) and others were dodecanoic acid (21.12%), hexadecanoic acid (11.58%), tetradecanoic acid (8.94%) and Octadecanoic acid (7.09%) methyl esters at retention time 13.0, 15.4, 20.8, 22.7 and 23.0 mins respectively. The fatty acid esters identified in the essential oil have been reported to have antibacterial and antifungal activities [1, 17-19] and these constituents might have contributed to the antimicrobial properties of *C. sordidum.*

TABLE 3: MINIMUM INHIBITORY CONCENTRATION (MIC) OF ETHANOL EXTRACT, ETHYL ACETATE AND HEXANE FRACTIONS OF *COMBRETUM SORDIDUM*

Extracts	Organisms	Conc. of extract/fraction in µg/mL							
		10000	2000	400	80	16	3.2	0.64	0.128
Hexane	*B. cereus*	+	+	+	+	+	+	+	+
	B. subtilis	+	+	+	+	+	+	+	+
	P. mirabilis	+	+	+	+	+	+	+	+
	S. typhii	-	-	-	+	+	+	+	+
	C. albicans	+	+	+	+	+	+	+	+
Ethylacetate	*B. cereus*	-	-	-	-	-	+	+	+
	B. subtilis	-	-	-	+	+	+	+	+
	P. mirabilis	-	-	+	+	+	+	+	+
	S. typhii	-	+	+	+	+	+	+	+
	C. albicans	-	-	-	+	+	+	+	+
Ethanol	*B. cereus*	-	-	+	+	+	+	+	+
	B. subtilis	-	-	-	+	+	+	+	+
	P. mirabilis	-	-	-	+	+	+	+	+
	S. typhii	-	-	-	+	+	+	+	+
	C. albicans	-	-	-	+	+	+	+	+

Key: + = Growth; - = No growth

TABLE 4: CHEMICAL COMPOSITION OF ESSENTIAL OIL OF *COMBRETUM SORDIDUM* LEAVES

Compounds	[a]RI	[b]Mm	[c]Mf	[d]Pa
Dodecanoic acid methyl ester	1481	214	$C_{13}H_{26}O_2$	21.12
Tetradecanoic acid methyl ester	1680	242	$C_{15}H_{30}O_2$	8.94
Hexadecanoic acid methyl ester	1878	270	$C_{17}H_{34}O_2$	11.58
7- Octadecenoic acid methyl ester	2085	296	$C_{19}H_{36}O_2$	51.26
Octadecanoic acid methyl ester	2077	298	$C_{19}H_{38}O_2$	7.09
Total				**99.99**

[a]Retention index; [b]Molecular mass; [c]Molecular formular; [d]Percentage area

CONCLUSION: The antimicrobial activities of *C. Sordidum* extracts corroborate the ethnomedicinal uses of this genus. The constituents of the extracts from the phytochemical analysis confirmed the reason for the antimicrobial activities of the plant against organisms used and therefore could be harnessed as a potent antimicrobial agent. Also the results obtained could form a good basis for further investigation in the discovery of new natural bioactive compounds. This report can be considered as the first scientific information on the phytochemical, antimicrobial properties and chemical composition of the essential oil constituents of *C. sordidum.*

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