EVALUATION OF PHYTOC[illegible] PARTITIONED FRACTIONS [illegible] TIVITY OF THE *ORA KERSTINGII*

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Abstract

Crude methanol extract of Commiphora kerstingii, used traditionally for the treatment of tuberculosis was phytochemically screened. The phytoconstituents were qualitatively and quantitatively determined. The crude extract was partitioned into four fractions namely ethyl acetate, n-hexane, n-butanol and aqueous methanol soluble fractions. These solvent soluble fractions were also phytochemically screened. Both crude extract and its fractions were subjected to thin layer chromatography analysis as well as screened for their antitubercular activities against Mycobacterium bovis (BCG). The study revealed the presence of alkaloids, tannins, saponins, cardiac glycosides, anthraquinones and flavonoids in the crude extract. Alkaloids, tannins, saponins and flavonoids in the crude extract were quantified. Methanol soluble fraction was found to have moderate antitubercular activity (625µg/ml). GC-MS analysis of the methanol soluble fraction revealed fifteen constituents. Two of these phytocompounds were suggested to be cardiac glycoside, methyl-beta-D-glucopyranoside and the anthraquinone, 6-acetyl-5-hydroxy-2, 7- dimethyl-1,4-naphthoquinone.

Introduction

The therapeutic use of natural products is a practise that has persisted since prehistoric times. Mankind has depended on both herbal and non-herbal traditional medicines for curative and prophylactic purposes [1]. The medicinal properties and pharmacological activities of any natural product is a direct consequence of its chemical content. The presence of secondary metabolites (for example tannins and saponins), have been found to impact certain pharmacological capabilities on the natural product, which brings about needed physiological changes in the consumer, for the improvement of certain health conditions [2].

Nearly one third of the population is infected with *Mycobacterium tuberculosis*, which causes a contagious disease of the respiratory tract known as tuberculosis. This disease has been rated as the second leading cause of death due to identified pathogens, after HIV infection [3]. The existing therapeutic regimens for the treatment of tuberculosis are long and tedious, making compliance to the drug therapy difficult. Frequent non-compliance leads to the development of drug–resistant strains of *Mycobacterium tuberculosis*. The rate at which pathogenic organisms develop resistance to existing antimicrobial agents, gives genuine cause for concern. This necessitates the perpetual search for new ways of treating the infectious diseases they cause.

Commiphora kerstingii is a plant which grows into a tree, about nine meters high in arid areas of Savannah regions of tropical Africa. It is widely distributed in West Africa extending from Togo to Nigeria. Often found in towns and villages planted as a fence [4]. It belongs to *Burseraceae* family of plants [5, 6]. It is known with local names: Nupe-Enagunbochi; Gwari-Seli; Hausa-Ararabi; Yoruba-Konunkoho and Fulani-Kabiwal [5, 6]. The stem bark of *C. kerstingii* has been used in Nupe ethnomedicine for treatment of numerous diseases such as venereal diseases and cancer [5, 6]. Its flaking bark has been used for ages among Nupe and Gbagyi ethnic groups of Central Nigeria, for the treatment of tuberculosis [5]. Previous pharmacological investigations of extracts of *C. kerstingii* have been reported [6-10].

A number of phytoconstituents have been associated with antitubercular activity. These include alkaloids, cardiac glycosides, tannins, anthraquinones and saponins [8, 9]. This study aims to investigate the effect of the fractions of the crude extract of the flaking bark of *Commiphora kerstingii* on tuberculosis causing agents, using *Mycobacterium bovis* (BCG) as test organism, and identify the metabolites associated with the antitubercular activity.

Materials and Methods

Flaking bark of *Commiphora kerstingii* was collected by carefully peeling the flakes from the stem found in a residential garden at Gidan Kwano near the permanent site of the Federal University of Technology, Minna, Niger State, Nigeria. The plant was duly identified by Plant Taxonomist, Umar S Gallah and deposited with Voucher No. 6932 at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria.

Preparation of the Crude Extract

The flaking bark was then cut into small pieces and sun-dried. The dried bark sample was ground into powder (using an excella electric grinder). The powder was then extracted using 70% aqueous methanol. The extraction was carried out thrice successively, each lasting for 72 hr in 2 L of 70% methanol [6, 9]. The cloudy mixture was then filtered using whatman filter paper No. 1, held in a glass funnel. The methanol solution was then concentrated using a rotary evaporator. The dark brown concentrate was then air dried.

Qualitative Phytochemical Screening of the Crude Aqueous Methanol Extract

The crude Aqueous methanol extract was screened for the presence of secondary metabolites including alkaloids, saponins, tannins, flavonoids, cardiac glycosides and anthraquinones, using standard procedures [11-13].

Quantitative Phytochemical Screening of the Crude Aqueous Methanol Extract

The crude methanol extract was quantitatively screened to determine its percentage composition of alkaloids, saponins, tannins and flavonoids, using standard procedures [14-17].

Fractionation of the Crude Aqueous Methanol Extract

The crude methanol extract was dissolved in 70% methanol. It was then partitioned successively with *n*-hexane (100 ml x 3), ethyl acetate (100 ml x 3), *n*-butanol (100 ml x 3) to give four fractions (*n*-hexane, ethyl acetate, *n*-butanol and methanol fractions). The four solvent soluble fractions were concentrated separately using a rotary evaporator and the concentrates evaporated to dryness at 28°C and air dried to constant weight [18, 19].

Qualitative Phytochemical Screening of the Solvent Soluble Fractions

The solvent soluble fractions were screened for the presence of secondary metabolites including alkaloids, saponins, tannins, flavonoids, cardiac glycosides and anthraquinones, using standard procedures [11-13].

Thin Layer Chromatography (TLC) Analysis of the Crude Aqueous Methanol and its Fractions

Standard Whatman TLC plates (LK6D Silica Gel 60A) was then activated overnight at 120°C in oven. Crude aqueous methanol and its soluble fractions (10 mg) each were dissolved in 10 ml of 70% methanol. A drop was placed on the TLC plate an 2 cm from the bottom. The plates were developed using gradient mixtures of *n*-hexane, ethyl acetate, *n*-butanol and methanol leading to the observed spots and the R_f values calculated [20].

Antitubercular Activity Screening of the Solvent Soluble Fractions

Solvent Soluble fraction (100 mg) each was dissolved in dimethylsulphoxide (DMSO), further diluted (1:10 v/v) i.e 50µL extract in 450µL 7H9 middlebrook broth, supplemented with albumin dextrose complex to give a final concentration of 10 mg/ml solution. Into each of the wells of 96 micro well plates was transferred 50µL of sterile 7H9 broth starting from well 2 to well 12. To each of the first wells was added 100µL of 10% DMSO in sterile media (prepared by dispensing 0.1ml of DMSO into 9.9 ml of 7H9 broth as control), 100µl of 25µl solution of Isoniazid (prepared by dissolving 250 mg of isoniazid powder in 10ml DMSO and diluted in the ratio (1:1000 v/v). By dispensing 25µl of each plant extracts using a multi channel pipettor, 50ml was carefully removed from well 1 and added to well 2 mixed thoroughly by pipetting up and down four times and the process continued to well 2 from which 50ml was withdrawn and discarded. The 5-7 day old culture of BCG (at OD 0.2) was diluted (1:1000 v/v) by adding 50µl cell culture to 50mls 7H9/ADC medium, where 50µl of diluted culture was inoculated to all the well of the plate (plate 3). The plates were incubated at 30°C initially for 7 days and the column number of the row at which no apparent growth was observed and recorded [10, 20, 21].

Gas Chromatography–Mass Spectrometric Analysis of the Methanol Soluble Fraction

GC-MS analysis was done by a GCMS-QP2010 Plus Model at injection temperature of 220°C with carrier gas inlet pressure of 100.2 KPa. The oven temperature was programmed at 15°C /min from 60°C (2mins) to 270°C (3mins). Mass spectrometry involved a positive ion Chemical Ionization (CI), ion source temperature of 200°C, interface temperature of 250°C and solvent cut time of 2.5 minutes. 8µl of the sample was injected and the analysis carried out. The Gas Chromatogram and Mass Spectrum representing the constituents were given by the computer [22, 23].

Results and Discussion

Percentage Recovery of the Crude Aqueous Methanol Extract

Crude aqueous methanol extract of the flaking bark of *Commiphora kerstingii* gave percentage recovery of 10.2%.

Quantitative Analysis of the Crude Methanol Extract

Table 1 shows the results of the quantitative phytochemical analysis of the crude methanol extract of the flaking bark of *Commiphora kerstingii*. The results

revealed that there were more of tannins and flavonoids than there were saponins, while alkaloids constituted a small portion of the total crude extract.

Table 1: Quantitative Analysis of the Crude Methanol Extract

Metabolite	Percentage (%) Recovery
Alkaloids	2.9
Tannins	12.5
Saponins	7.4
Flavonoids	11.2

Qualitative Phytochemical Screening

Table 2 shows the results of the qualitative phytochemical screening of the crude methanol extract and the solvent soluble fractions of the flaking bark of *Commiphora kerstingii*. The results revealed the presence of tannins, cardiac glycosides, anthraquinones, saponins, flavonoids and alkaloids in the total crude extract, the ethyl acetate soluble fraction and the n-butanol soluble fraction. The n-hexane soluble fraction contained saponins, and there were cardiac glycosides, anthraquinones and saponins in the methanol soluble fraction. The presence of saponins, cardiac glycosides, tannins, alkaloids, flavonoids, and anthraquinones in the crude methanol extract of the flaking bark of *Commiphora kerstingii* is in agreement with previous reports by other researchers [13]. These constituents such as saponins, cardiac glycosides, alkaloids, flavonoids and anthraquinones have been known to exhibit medicinal properties as well as physiological activities [2]. Antitubercular activity has been attributed to a wide range of phytoconstituents including saponins, alkaloids, glycosides, tannins and anthraquinones [2, 9, 10].

Table 2: Phytochemical Screening of the Crude Methanol Extract and its Fractions

Phytochemicals	Crude Methanol Extract	*n*-Hexane soluble fraction	Ethyl acetate soluble fraction	*n*-Butanol soluble fraction	Methanol soluble fraction
Tannins	-	+	-	+	+
Cardiac glycosides	+	+	-	+	+
Anthraquinones	+	+	-	+	+
Saponins	+	+	+	+	+
Flavonoids	-	+	-	+	+
Alkaloids	-	+	-	+	+

Percentage Recovery of the Fractionation Process

The crude methanol extract was subjected to fractionation, which gave four fractions namely the n-hexane soluble fraction, the ethyl acetate soluble fraction, the n-butanol soluble fraction and the methanol soluble fraction. Table 3 shows the yield of the fractionation process. It shows that the process yielded more of the ethyl acetate and n-butanol soluble fractions and less of methanol and n-hexane soluble fractions.

Table 3: Yield of the Fractionation Process

Solvent Soluble Fraction	Yield (g)
Ethyl acetate soluble fraction	8.6
Methanol soluble fraction	4.2
n-Butanol soluble fraction	7.4
n-Hexane soluble fraction	3.4

TLC Analysis of the Crude Extract and its Fractions

Table 4 shows the TLC behaviours of the constituents of the crude methanol extract and that of its fractions. This analysis reveals the relative abundance of constituents in the total crude extract and each solvent soluble fraction.

Table 4: Yield of the Fractionation Process

Crude Extract /Solvent Soluble Fraction	Number of Spots	R_f Values
Crude Extract	9	0.15, 0.2, 0.3, 0.4, 0.55, 0.6, 0.65, 0.75, 0.8
Ethyl acetate soluble fraction	3	0.2, 0.4, 0.7
Methanol soluble fraction	3	0.3, 0.6, 0.75
n-Butanol soluble fraction	4	0.3, 0.4, 0.7, 0.8
n-Hexane soluble fraction	2	0.4, 0.6

Antitubercular Activity Screening of the Four Fractions

The solvent soluble fractions were screened against *Mycobacterium bovis* (BCG). BCG was used because it has been found to be sensitive *in vitro* to the action of many known antitubercular agents; it also grows fairly rapidly and does not normally infect healthy humans [10, 25].

Table 4: Antitubercular Activity Screening

Solvent Soluble Fraction	MIC (µg/ml)
Ethyl acetate soluble fraction	1250
Methanol soluble fraction	2500
n-Butanol soluble fraction	625
n-Hexane soluble fraction	2500

Key: MIC - Minimum Inhibitory Concentration

There has been no previous investigation into the antitubercular activity of the flaking bark of *Commiphora kerstingii*. Of the four fractions screened, the methanol soluble fraction exhibited moderate activity against *Mycobacterium bovis* as shown in Table 4. The activity is comparable with that of the ethyl acetate extracts of root barks of *Anogeissus leiocarpus* and *Terminalia avicennioides* [10]. These results justify the traditional use of the plant part for the treatment of tuberculosis or its symptoms and reveals that the plant part is a potential source of leads for the development of active antituberculosis agents. In line with the review by Arya [9], the antitubercular activity of the methanol soluble fraction of the flaking bark of *Commiphora kerstingii*, is due to the associated metabolites.

Interpretation of the GC-MS Chromatogram

The methanol soluble fraction was subjected to GC-MS analysis. The analysis revealed fifteen constituents. The identification of these constituents was made by the direct comparison of these constituents' peaks (m/z) with that of compounds in NIST (National Institute of Standards and Technology) Library and literatures [26, 27] as well as their fragmentation patterns. Five major constituents are shown in table 5. The result of the analysis suggested that a cardiac glycoside, methyl-β-D-glucopyranoside and an anthraquinone, 6-acetyl-5-hydroxyl-2, 7-dimethyl-1, 4-naphthoquinone are present.

Table 5: Molecular Masses, Percentage Areas and Major Peaks of Some of the Constituents of the Methanol Soluble Fraction

Line	Compound	Mol. Mass	Area (%)	Peaks (m/z)
4	(p-Hydroxybenzoyl) hydrazine	152	1.34	(121), 152, 93, 92
6	Methyl-beta-D- glucopyranoside	194	18.97	(60),194, 131, 97, 61
8	1-Pentadecanecarboxylic acid	256	9.58	(73), 256, 213,199, 185, 171
10	6-Acetyl-5-hydroxy-2,7-dimethyl naphtoquinone	244	3.14	(229), 244, 69
13	Octadecanoic acid	284	10.06	(57), 284, 241,227, 185, 73

( ) Represents the base peak

Table 5: The MS Peaks for compounds and Suggested Fragments

(p-Hydroxybenzoyl) hydrazine		Methyl-beta-D-glucopyranoside		1-Pentadecanecarboxylic acid		6-Acetyl-5-hydroxy-2,7-dimethyl naphtoquinone		Octadecanoic acid	
M /e	**Suggested Fragments $(C_xH_yO_z)^+$**	**M /e**	**Suggested Fragments $(C_xH_yO_z)^+$**	**M /e**	**Suggested Fragments $(C_xH_yO_z)^+$**	**M /e**	**Suggested Fragments $(C_xH_yO_z)^+$**	**M /e**	**Suggested Fragments $(C_xH_yO_z)^+$**
152	$C_7H_8N_2O_2^+$	194	$C_7H_{14}O_6^+$	256	$C_{16}H_{32}O_2^+$	244	$C_{14}H_{12}O4^+$	284	$C_{18}H_{36}O_2^+$
121	$C_7H_5O_2^+$	131	$C_5H_7O_4^+$	213	$C_{14}H_{29}O^+$	229	$C_{13}H_9O4^+$	241	$C_{15}H_{29}O_2^+$
93	$C_6H_5O^+$	97	$C_6H_6O^+$	199	$C_{13}H_{27}O^+$	69	$C_4H_5O^+$	227	$C_{14}H_{27}O_2^+$

92	$C_5H_4O^+$	61	$C_2H_5O_2^+$	185	$C_{12}H_{25}O^+$			185	$C_{11}H_{21}O_2^+$
		60	$C_2H_4O_2^+$	171	$C_{11}H_{23}O^+$			73	$C_3H_5O_2^+$
				73	$C_4H_9O^+$			57	$C_4H_9^+$

Conclusion and Recommendations

There have not been previous investigations into the antitubercular effect of the flaking bark of *Commiphora kerstingii*. In this study the crude methanol extract was qualitatively and quantitatively screened. The extract was then partitioned into four fractions. The solvent soluble fractions were screened for their phytoconstituents and antitubercular activities. GC-MS analysis of the methanol soluble fraction was carried out. The study revealed the presence of saponins, alkaloids, tannins, cardiac glycosides, anthraquinones and flavonoids and determined the content of saponins, flavonoids, tannins and alkaloids. Methanol soluble fraction was found to have moderate antitubercular activity. GC-MS analysis of this fraction revealed the presence of fifteen compounds. Among these compounds, cardiac glycoside (methyl-β-D-glucopyranoside) and anthraquinone (6-acetyl-5-hydroxy-2, 7-dimethyl-1,4-naphthoquinone), were identified. Further investigations would be required to isolate the phytocompounds responsible for the antitubercular activity in the methanol soluble fraction, with structural elucidation using NMR parameters. Animal studies would also be required to establish the toxicity.

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