Antimicrobial activity and determination of quercetin and ellagic acid in stem bark of Sterculia setigera, Del.

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Abstract

Sterculia setigera stem bark extracts (petroleum ether and ethanol with water) and fractions of aqueous ethanol (chloroform, ethyl acetate n-butanol and water), were examined for their antimicrobial activity using cup-plate-agar diffusion method against four standard bacterial species; two gram-positive bacteria viz., staphylococcus aureus (ATCC 25923) and Bacillus subtilis (NCTC 8236), two gram-negative bacterial strains Pseudomonas aerugionosa (ATCC 27853) and Escherichia coli (ATCC 25922), and two standard fungal species ; Candida albicans (ATCC 7596) and Asparagellus nigar (ATCC 9763). The results obtained from the diameters measurement of the growth inhibition zones of microorganisms showed that aqueous ethanol extract exerted intermediate to significant antimicrobial activity against tested organisms ranging from 13.5-25.5mm. Ethyl acetate, n-butanol and aqueous fractions were found to have broad spectrum effect against standard organisms. Ethyl acetate fraction exhibited significant antimicrobial activity with the highest zones of inhibition ranging from 27-32 mm against tested bacteria and 21mm against fungi.

Two phenolics of biologically and pharmacologically important; quercetin, ellagic acid were identified using high-performance liquid chromatography (HPLC) method. The characteristic peaks in stem bark extract were identified by comparing retention time of the sample solution with those of reference standards. The study confirmed the presence of quercetin and ellagic acid in Sterculia setigera stem bark.

Key words: Sterculia setigera, antimicrobial activity, quercetin, ellagic acid

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Introduction

Sterculia setigera Del., English name karaya gum tree, belongs to the family Sterculiaceae. It is a deciduous savanna tree up to 15m high. Bark grey purple, slash red with paler streaks, exuding white gum and watery sap. It grows wildly in Sudan in the Blue Nile area, Kassala, Northern Darfur, southern Kordofan and Red Sea hills. Infusion prepared from stem bark is used to treat jaundice in Southern Blue Nile district of Sudan. Bark as a mixture is macerated and used against dysentery by some tribes in central Nigeria. Ethanol stem bark extract of Sterculia setigera was shown to have antibacterial activity on Pseudomonas aeruginosa with a minimum inhibitory concentration of 2.0 mg/ml and Bacillus subtilis with a minimum inhibitory concentration of 1.0 mg/ml. Crude methanolic extract of stem bark showed some activity against Mycobacterium tuberculosis at a minimum inhibitory concentration of 2500 µg/ml.

Phytochemical analysis of Sterculia setigera stem bark reveals the presence of flavonoids, alkaloids, saponins, volatile oil. For quantitative and qualitative analysis of specific analytes in a plant extract a separation method has to be employed before detection. Different types of chromatographic separation have been used in the analysis of flavonoids. High-performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants. HPLC method was used in this study to detect the presence of quercetin and ellagic acid in Stem bark of Sterculia setigera.

Materials and Methods

Collection of Plant and Identification

Sterculia setigera plant was collected from Southern Kordofan state, western of Sudan in November 2010 and identified at Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Research Centre, Khartoum, Sudan. Voucher specimens were deposited at the herbarium of the institute. Collected plant parts were cleaned, chopped into pieces, air dried under shade for 4 weeks, and then coarsely powdered using a clean electric blender then carefully stored.

Preparation of plant extracts

1kg of the stem bark powder of Stericula setigera plant was successively extracted with petroleum ether and 80% ethanol using shaker extractor apparatus. Extraction carried out for about three days with daily filtration and evaporation the solvent for petroleum ether and five days for ethanol. Solvents were evaporated under reduced pressure using rotatory evaporator. Finally extracts allowed to dry in air in petri- dishes and the yield percentages were calculated as follows:

Weight of extract obtained / weight of plant sample X100

Petroleum ether yielded 0.9% whitish green powder while aqueous ethanol gave reddish brown gummy powder 9.8%.

Excess petroleum ether was added to 100g of aqueous ethanol extract and shacked several times and then left overnight at room temperature. Then filtered and the solvent evaporated in vacuo .Then defatted aqueous ethanolic extract from stem bark of Stericula setigera plant was dissolved in water and subjected to successive extraction in a separatory funnel with solvents in ascending polarity to give four fractions. The aqueous solution was first re-extracted with chloroform, then ethyl acetate and finally n-butanol. The residue is aqueous extract. Solvents were evaporated under reduced pressure. Aqueous layer was lyophilized using freeze-drier machine till dryness and the yield percentages of each fraction were calculated. The fractions obtained were: chloroform fraction which is yellowish green oily powder with yield percentage 0.7%, ethyl

acetate fraction is 0.9% pale brown powder, n-butanol gave reddish brown powder 4.0% and aqueous fraction
is brown powder with yield percentage 21%.

In vitro testing of extracts and fractions for antimicrobial activity

petroleum ether and 80%ethanol extracts of Sterculia setigera stem bark and fractions from aqueous ethanol (chloroform, ethyl acetate n-butanol and water), were tested against four standard bacteria species: two Gram-positive bacteria viz., Bacillus subtilis (NCTC 8236) and staphylococcus aureus (ATCC 25923), two Gram-negative bacterial strains Escherichia coli (ATCC 25922) and Pseudomonas aerugionosa (ATCC 27853), and two standard funal species: Candida albicans (ATCC 7596) and Asparagellus nigar (ATCC 9763) using the cup-plate-agar diffusion method (Kavaragh 1972) with some minor modifications. From each powdered extracts and fractions a 10% solution was prepared as follows: Petroleum ether extract dissolved in petroleum ether, chloroform fraction dissolved in mixture of petroleum ether and methanol with ratio 1:2, ethanol extract in methanol and aqueous extract dissolved in water.
The standard bacterial and fungal strains used in this study were obtained from the
Department of Microbiology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Research Centre, Khartoum, Sudan.
To assess the antibacterial activity of the prepared extracts, 6.4ml of each of the four standardized bacterial stock suspensions (10^8-10^9 C.F.U./ml) were thoroughly mixed with 640 ml of sterile melted nutrient agar which was maintained at 45ºC. 20 ml of aliquots of the inocubated nutrient agar were distributed into sterile 32 petri-dishes. The agar was left to set and in each of these resulting plates, which were divided to two halves, two cups in each half (10 mm in diameter) were cut using a sterile cork borer (No.4). Each one of the 64 halves was designed for one of the extracts. The agar disks were removed. Alternate cups were filled with 0.1 ml samples of each of the extracts using transfer pipette adjustable volume automatic microtitre pipette, and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37 ºC for 24 hours.
Two replicates were carried out for each extract against each of the test organisms
simultaneously; (positive) controls involving the addition of the respective solvents instead of the

extracts were carried out separately. After incubation the diameters of the resultant growth inhibition zones were measured and averaged.

The same method used above for bacteria was adopted for testing fungi. However, sabouraud dextrose agar was used instead of nutrient agar and the inoculated medium was incubated at 25 ºC for two days for the Candida albicans and three days for Aspergillus niger.

An antibacterial reference drug (Ciprofloxacin tablet) and an antifungal reference drug (Itraconazole tablet) were prepared in suspensions of four concentration (40, 20, 10, and 5mg/ml)) using sterile distilled water; to evaluate their antibacterial
and antifungal activity against the tested standard organisms, using the cup plate agar method previously described. Removed cups were filled with 0.1 ml samples of each of the four concentrations of the reference drugs using Transferpette adjustable volume automatic microtitre pipette, and allowed to diffuse at room temperature for two hours.

High-performance liquid chromatography (HPLC) analysis

Powdered stem bark were extracted with 90% methanol. The extract was hydrolyzed using 2M HCl at 100°C for 30min.Then the hydrolysate was extracted with ethyl acetate using a separatory funnel. Ethyl acetate layer was separated, dried and dissolved in methanol. The methanolic solution was filtered through membrane filter.

In order to identify four phytochemical markers in stem bark of Sterculia setigera, solutions of equal concentration (10 µL each) of samples and reference standards were automatically injected into an Alliance HPLC system (e2695 Separation module, Waters, USA) equipped with a PDA detector. The HPLC system is equipped with a Hypersil 5.0µ C18 (ODS) column (150 × 4.6 mm, Phenomenex, USA). The mobile phase used was consists of 0.5% formic acid in water(A) and methanol (B) at a flow rate of 1.0 mL/min. The gradient elution program was as follows: 5 min 90% A, 5min 70% A, 5 min 30% A, 5 min 2% A.

Detection was carried out in scanning mode (3D channel) and the specific wavelengths were set at:

254 nm for ellagic acid and quercetin.

280 nm for gallic acid.

326 nm for chlorogenic acid.

The column temperature was maintained at 30°C. The identities of compounds were established by comparing retention time of the sample solution with those of standard solutions. The developed method has been validated as per the International Conference on Harmonization guidelines (ICH Q2B, 1996).

Results and Discussion

Sterculia setigera stem bark extract (petroleum ether and ethanol with water) were examined for their antimicrobial activity using cup-plate-agar diffusion method against four bacteria species, (staphylococcus aura, Bacillus subtilis, Pseudomonas aerugionosa and Esherichia coli, and two fungal species; (Candida albican and Asparagellus nigar. Most extracts had high activity against tested organisms. 80% ethanol extract exerted sensitive activity against Bacillus subtilis(25.5mm) and Pseudomonas aeruginosa(16.5mm). It showed intermediate activity against Staphylococcus aureus(13.5mm) and Escherichia coli(15mm) and sensitive with both tested fungi(17,17.5mm).

The result obtained revealed that ethyl acetate fraction of Sterculia setigera stem bark possess the highest activities against both fungi and bacteria. Its activity ranged from27-30mm against bacteria and 21mm against fungi (sensitive). It showed more activity against gram-negative bacterial strains than gram-positive. Its activity against gram-negative bacterial strains and fungal strains is higher than reference drug with concentration of 20, 10 and 5 mg/ mL.

Table 1: Diameters (mm) of the resultant growth inhibition zones for antibacterial activity of stem bark of Sterculia setigera extract by polar and non polar solvents

Extracting solvent	E.C	P.S	B.S	S.a
Petroleum ether	-	-	-	-
80 % ethanol	15	16.5	25.5	13.5

Key: B.s =Bacillus subtilis, S.a=Staphylococcus aureus, E.c=Escherichia coli , Ps =Pseudomonas aeruginosa. >15mm=sensitive, 12-15mm=intermediate, <12mm= resistant, (-) = No inhibition.

Table 2: Diameters in mm of the resultant growth inhibition zones for antifungal activity of stem bark of Sterculia setigera extracts by polar and non polar solvents

Extracting solvent	Ca.a	As.n
Petroleum e	-	-
80 % ethanol	17.5	17

Key: Petroleum e=petroleum ether, Ca.a =Candida albicans, As.n = Aspergillus nigar

Table 3: Diameters of the resultant growth inhibition zones for antibacterial activity of the fractions obtained from fractionation of the stem bark ethanol extract of Sterculia setigera

Fraction	Diameters of inhibition zones (mm)			
	E.coli	P.S	B.S	S.a
Chloroform	16	12.5	13.5	-
Ethyl acetate	27	28	29	32
n-butanol	17	16	14	15
Aqueous	24.5	20.5	26.5	13

Table 4: Diameters of the resultant growth inhibition zones for antifungal activity of the fractions obtained from fractionation of the stem bark ethanol extract of Sterculia setigera

Fraction	Diameters of inhibition zones (mm)	
	Ca.a	As.n
Chloroform	-	-
Ethyl acetate	21	21
n-butanol	16	15
Aqueous	17	-

Table 5: Diameters of the resultant growth inhibition zones for antibacterial activity of standard (Ciprofloxacin tablet) and antifungal activity of standard (Itraconazole tablet)

Conc mg/ mL	Bacteria				Fungi	
	E.C	PS	B.S	Sa	Ca	A.u
40	40	41	30	36	22	-
20	35	37	27	30	20	-
10	33	35	23	27	17	-
5	30	31	20	23	15	-

HPLC method was used in this study to detect the presence of quercetin and ellagic acid in Stem bark of Sterculia setigera. HPLC chromatograms of standard quercetin and ellagic acid were shown in figures 1 and 2. HPLC fingerprinting analysis of stem bark is shown in figures 3, 4 and 5 and Tables 6, 7 and 8. Spectra revealed the presence of ellagic acid(1.57%) and quercetin(0.16%) among 25 well-separated compounds at 254nm.There are 21 compounds at 278nm and 12 compounds at 326 nm.

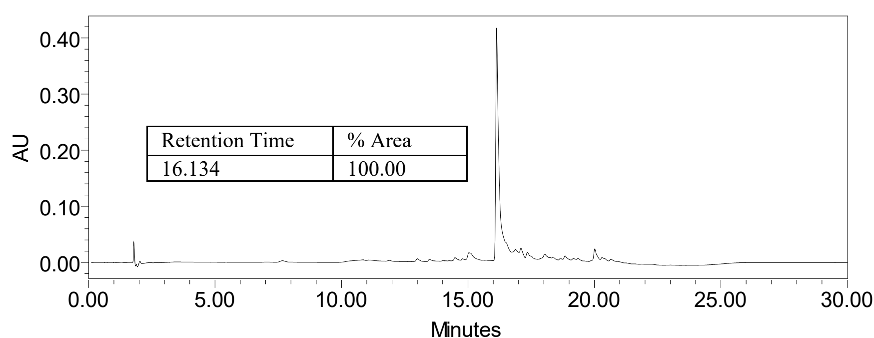

Figure 1: HPLC Chromatogram of Standard quercetin.

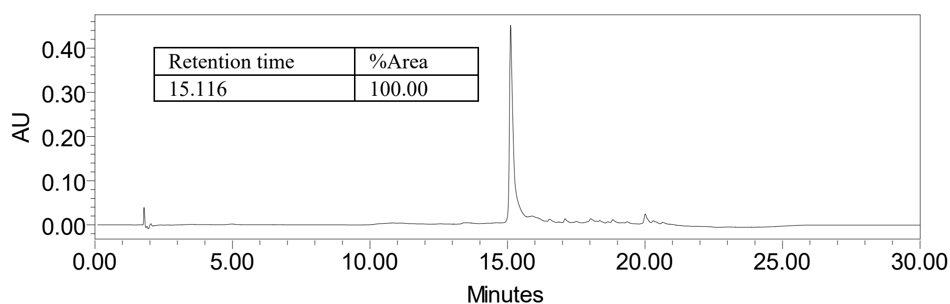

Figure 2: HPLC chromatogram of standard ellagic acid.

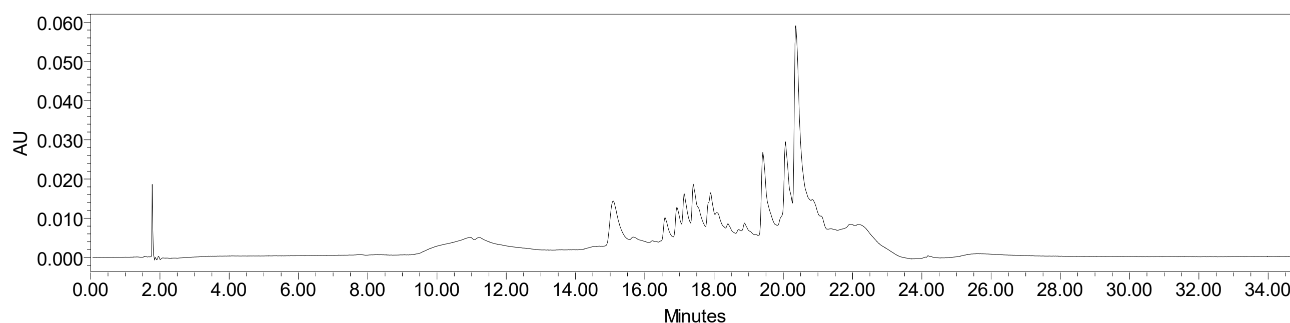

Figure 3: HPLC chromatogram of ethyl acetate extract of Sterculia setigera stem bark at 254nm.

Table 6: HPLC analysis of ethyl acetate extract of Sterculia setigera stem bark at 254nm

Peak No.	Name	Retention Time	% Area
1		10.037	0.49
2		10.950	2.05
3		11.218	0.73
4	Ellagic acid	15.117	1.57
5		15.657	0.12
6	Quercetin	16.194	0.16
7		16.582	1.62
8		16.926	0.41
9		17.139	2.27
10		17.557	2.50
11		17.833	1.57
12		18.080	4.59
13		18.404	2.86
14		18.700	1.59
15		18.883	3.81
16		19.302	1.11
17		19.404	4.05
18		20.059	6.16
19		20.352	4.06
20		20.685	5.90
21		21.693	1.01
22		21.904	2.48
23		24.078	0.02
24		24.182	0.03
25		25.937	18.88

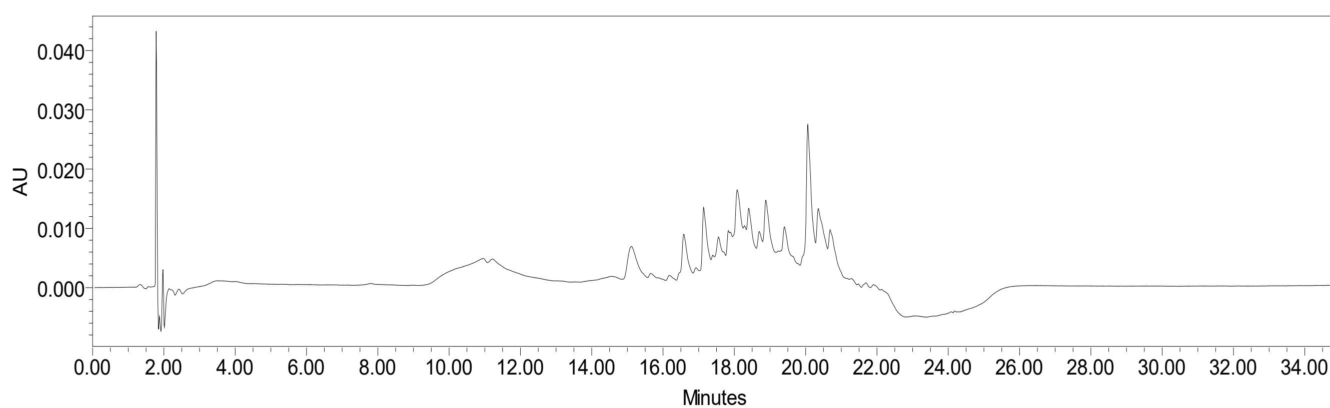

Figure 4: HPLC chromatogram of ethyl acetate extract of Sterculia setigera stem bark at 278nm.

Table 7: HPLC analysis of ethyl acetate extract of Sterculia setigera stem bark at 278nm

Peak No.	Retention Time	% Area
1	10.038	0.94
2	10.941	4.02
3	11.216	1.36
4	15.086	6.90
5	15.662	0.49
6	16.222	0.12
7	16.584	2.21
8	16.928	2.78
9	17.137	4.19
10	17.401	7.06
11	17.900	4.75
12	18.069	3.02
13	18.401	1.34
14	18.711	0.49
15	18.879	1.43
16	19.408	8.84
17	20.059	9.50
18	20.358	28.99
19	21.998	0.67
20	22.244	3.87
21	24.177	0.21

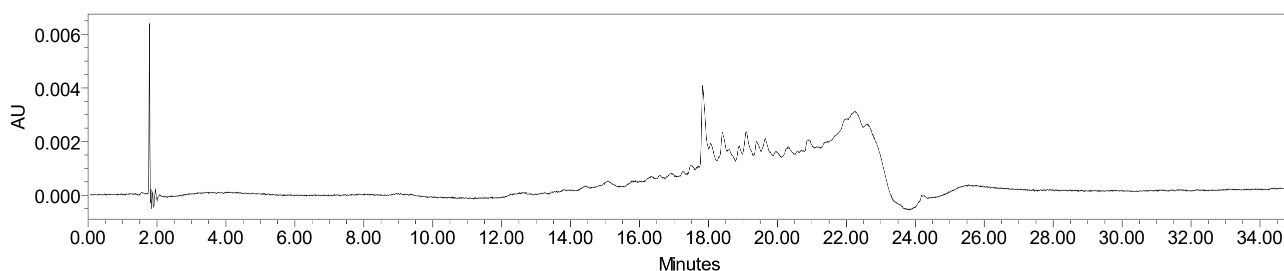

Figure 5: HPLC chromatogram of ethyl acetate extract of Sterculia setigera stem bark at 326nm.

Table 8: HPLC analysis of ethyl acetate extract of Sterculia setigera stem bark at 326nm

Peak No.	Retention Time	% Area
1	17.471	0.44
2	17.832	5.76
3	18.062	1.47
4	18.403	1.44
5	18.895	0.93
6	19.087	2.46
7	19.402	1.19
8	19.648	1.41
9	20.305	0.82
10	20.885	0.90
11	22.235	3.97
12	25.543	30.67

Conclusion

Stem bark of Sterculia setigera proved to exert a potent in vitro antimicrobial activity. The study confirmed the presence of quercetin and ellagic acid in the stem bark extract. Future research might involve isolation of active compounds and biosynthetic pathway using isotopic radioactive tracers.

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