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Hydroxyl radical scavenging, toxicity and antimicrobial investigation of stem bark extracts of Sterculiar tragacantha Lindl (Malvaceae)

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***Abstract*— Plants contain natural products which have been recognized as building blocks for primary health care. *Sterculiar tragacantha* Lindl, used traditionally in Nigeria for the treatment of boils, diarrhea, gonorrhea, edema, gout, whitlow, fevers and malaria is one of such plants. The prevalence of drug resistant pathogenic microbes as well as the oxidative damage to cells by excess free radicals that lead to degenerate diseases, constitute major health hazards to man. In this regard, extracts of the stem bark of *S. tragacantha* Lindl was investigated for their toxicity to brine shrimps and ability to scavenge hydroxyl radical and inhibit microbial growth. Air dried and ground stem bark of the plant was extracted with methanol and partitioned in n-hexane and ethyl acetate. Hydroxyl radical scavenging assay of the three extracts obtained revealed that the percentage inhibition for all the extracts was above 90% at 0.625 mg/ml which is higher than that of one of the antioxidant standard (ascorbic acid) used for the assay. The ethyl acetate and methanol extracts were toxic with LC_{50} value of 487.03 µg/ml and 482.80 µg/ml respectively, while the hexane was non toxic with LC_{50} value >1000 µg/ml in the brine shrimp lethality test. Bioactive compounds have been found to be often toxic to shrimp eggs. All the extracts demonstrated activity against the ten microbes: *Staphylococcus aureus, Esherichia coli, Bacillus subtilis, Pseudomonas aerugunosa, Salmonella typhi, Klebsiellae pneumonia, Candida albicans, Aspergillus niger, Penicillum notatum, Rhizopus stolonifer* used in the antimicrobial assay. Preliminary phytochemical screening revealed the presence of saponin, tannins, alkaloids, phenols, cardiac glycosides, resins and antraquinones as the secondary metabolites present in the extracts.**

The ability of the extracts to scavenge hydroxyl radicals and inhibit growth of microorganisms makes the plant a potential source of antioxidant and antimicrobial compounds and also validates the ethno medicinal use of *Sterculiar trangacantha* Lndl.

Keywords— Phytochemicals, hydroxyl radical, toxicity, antimicrobial, *Sterculiar tragacantha*

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Introduction

Natural products commonly understood to refer to herbal concoction, dietary supplement, traditional medicine or alternative medicine either of pre-biotic origin or originating from microbes, plants or animals sources is now recognized by the World Health Organization (WHO) as a building block for primary health care (Akerele, 1988). They include such classes of compounds as alkaloids, terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, etc which are also constituents of many plants. They provide treatments for pain, palliatives, or curatives for a variety of maladies (Burkill, 1997,Teijo et al., 2004).

The tree *Sterculiar tragacantha* Lindl belongs to the family Malvaceae. It has been reported that in many West African countries: Burkina Faso, Cameroon, Ghana, Guinea, Ivory Coast, Nigeria and Sierra Leone, the leaves, bark, shoots and seeds are used for the treatment of diarrhea, dysentery, arthritis, rheumatism, edema, gout and whitlow. The methanol leaf extract of *S. tragacantha* Lindl has been shown to possess analgesics activity and infusions of the leaves is used to treat malaria. *Sterculia* species have been extensively used in traditional medicine in various countries. *S. lychnophora* is used as herbal tea in Cambodia; *S. macrophylla* is used as an aphrodisiac in Java. In Australia, *S. quadrifolia* is used as peanut tree while in Thailand, it is used to treat fever. *S. shillinglawii* is also said to treat fever while in India, *S. urens* and *S. villosa* are used to treat fistula and night fever. Phytochemical investigation of *S. foetida* Linn's as antioxidant and antimicrobial agents has been reported.(Burkill, 1997, Manivannan et al., 2011). Not much report of chemical composition and pharmacological activity of *S. tragacantha* was found in literature.

The prevalence of drug resistant pathogenic microbes has made infectious diseases (bacterial, fungal and viral) major health hazards. Futhermore, Oxidative reactions initiated by excess free radicals have been shown to damage cells leading to degenerate diseases such as : premature aging, diabetes, tumors, cancer, cirrhosis, cardiovascular, nervous, rheumatic and pulmonary disorders etc. These also constitute serious health hazards to man. Although oxidation is important to aid biological activities or processes in living organism, production of polyunsaturated fatty acids generates reactive

oxygen species (ROS) such as hydroxyl radicals which in excess is injurious to man.

The need therefore for discovery of plants with antimicrobial and antioxidant activities cannot be overemphasized. In continuation of our research work on search for plants and compounds with antimicrobial and antioxidant activities (Oloyede and Farombi, 2010; Oloyede et al., 2010; 2011; Onocha et al., 2010; 2011), we now report on Hydroxyl radical scavenging, toxicity and antimicrobial investigation of stem bark extracts of *Sterculiar tragacantha* Lindl.

EXPERIMENTAL SECTION

MATERIALS: Chemicals and Reagents

All solvents used were BDH analar grade: hexane, methanol, ethyl acetate, chloroform, Fehling's solution A and B, 5 % Ferric chloride, concentrated tetraoxosulphate (VI) acid, Dragendroff's reagent, ammonia solution, copper acetate, Molisch reagent, glacial acetic acid, ammonia solution, copper acetate, phosphate buffered saline, standard BHA (2-tert-butyl-4-methoxyphenol or butylated hydroxyl aniole), dimethylsulphoxide (DMSO) (M&B, England), hydrogen peroxide, α-tocoperol, sodium chloride, butanol, hydrochloric acid, bismuth nitrate sodium hydroxide. However, Silica gel 60 - 230 microns (Merck, Germany) and ascorbic acid were obtained from Sigma Chemical Co (St Louis, MO) while Brine shrimp larvae eggs were obtained from Ocean Star International, Inc. Company, USA.

Equipment and apparatus

UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models), FT-IR Spectrophotometer. Soxhlet apparatus.

Plant collection, identification and Sample preparation

Fresh stem bark of *Sterculiar tragacantha* Lindl were collected from botanical garden of University of Ibadan, Ibadan in July 2012. Specimens were identified by Mr. Donatus Erastus and authenticated by Dr. Ayodele of the Department of Botany, University of Ibadan, Nigeria. The bark were chopped into pieces, air dried under shade for 21days, ground into mesh size and kept in a non-absorptive nylon for subsequent use.

Test Organisms:.Microorganism: *Staphylococcus aureus, Esherichia coli, Bacillus subtilis, Pseudomonas aerugunosa, Salmonella typhi, Klebsiellae pneumonia, Candida albicans, Aspergillus niger, Penicillum notatum, Rhizopus stolonifer* collected from the stock of the Dept. of Pharmaceutical Microbiology, Faculty of Pharmacy of University of Ibadan were used. They were maintained on nutrient agar slopes and kept in a refigerator at 4^{o}C. Nutrient broth (100 ml aliquot) were inoculated with the culture of test micro-organisms and then incubated for 24 hrs at 37^{o}C.

Reference Standards: Ascorbic acid, butylatedhydroxyanisole (BHA) and α-tocopherol for antioxidant activity; gentamicin and tioconazole for antimicrobial screening.

Extraction of the Plant Sample: Milled 1.30 kg of dried *S. tragacantha* Lindl stem bark was extracted with 5 litres of methanol using a Soxhlet apparatus. The extract obtained was collected, concentrated and partitioned with n-hexane to give the hexane soluble part of the crude extract. To the dried residue, ethyl-acetate was added again and stirred. This procedure was repeated until all components soluble in ethyl-acetate had been obtained. The remaining extract was tagged the 'Methanol extract' (MST), the two others were tagged 'n-hexane extract (HST) and ethyl acetate extracts' (EST), respectively.

Phytochemical Screening of Extracts: The extracts (HST, EST and MST) were screened for the presence of secondary metabolites like alkaloids, flavonoids, steroids, tannins, saponins, cardiac glycoside, phenols and resins (Harborne, 1985).

Antioxidant assay of the Extracts: Hydroxyl radical scavenging Activity of HST, EST and MST:

A rapid, simple and inexpensive method to measure antioxidant capacity of food and drugs involves the use of the free radical, 2, 2- Diphenyl-1-picrylydrazyl (DPPH) to test the ability of compounds to act as free radical scavengers or hydrogen. Other methods used include use of hydrogen peroxide and ferric thiocynate methods (Potterat, 1997, Oloyede and Farombi, 2010)

The ability of the extracts to scavenge hydroxyl radical was determined in this assay. A solution of hydrogen peroxide (2 μM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 285 nm using a spectrophotometer. The samples at 1.0 - 0.0625 mg/ml were added to H_2O_2 . The decrease in absorbance of H_2O_2 at 285 nm was measured spectrophotometrically after ten minutes against a blank solution containing the test sample in phosphate buffer saline without H_20_2 (control). All tests were run in triplicate and averaged (Oloyede and Farombi, 2010; Oloyede et al., 2010; Onocha et al., 2011).

Statistical Analysis: Absorbance measurements were evaluated as mean absorbance ± SD of triplicate analysis from which Percentage inhibition was calculated. Statistical analysis was performed by a one-way analysis of variance

(ANOVA) processed on SPSS 15 windows software for more than two means while Student's t-test was used for comparison between two means. Values of $p<0.05$ were taken to be statistically significant.

Toxicity analysis

Brine shrimp lethality test

Brine shrimp toxicity screening is a rapid *in vitro* assay that makes use of brine shrimp, Artemia species, also known as sea monkeys to assess toxicity to lower organisms. The advantages of the test are homogeneity in eggs and focuses on mortality of brine shrimps versus toxicant concentration in time (Meyer et al, 1982; Falope et al., 1993, Dvorak et al, 1999). The brine shrimp toxicity test was used to evaluate toxicity of the extracts (Meyer et al, 1982; Aiyelaagbe et. al, 2009). The shrimp's eggs were hatched in sea water for 48 hours at room temperature. The nauplii harvested shrimps were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations 1000, 100 and 10 µg/ml and incubated in triplicate vials. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 hours the survived larvae in the vials were counted. The concentration at fifty percent mortality of the larvae (LC_{50}) was determined using the Finney computer programme (Oloyede *et al*, 2010).

Antimicrobial Assay of Extracts HST, EST and MST

Sample Preparation

Each of the extracts (1000 mg) were weighed separately and dissolved in 5 ml of the solvent of extraction in other to obtain proper dissolution. From the 200 mg/ml solution, 2.5 ml was taken into another sample bottle and 2.5 ml of solvent was added to give 100 mg/ml, this was serially diluted until a 6.25 mg/ml concentration was obtained. Two other sample bottles contained the negative control (solvent of dissolution) and the positive control (Gentamicin 10 µg/ml for bacteria and Tioconazole 70% for fungi).

Agar diffusion: Pour plate method for bacteria

A culture of each organism (*S. aureus, E. coli, B. subtilis, P. aeruginosa, K. pneumoniae* and *S. typhi*) was prepared by adding 0.1 ml of each seperately to 9.9 ml of sterile distilled water to obtain 10ml of 1:100 dilutions. 0.2 ml was then taken into the prepared nutrient agar, cooled to 45^{o}C and poured aseptically into sterile petri-dishes and allowed to solidify. The prepared concentrations of the samples, positive and negative controls were introduced into drilled holes in the cultured microbes with syringes after 45minutes, allowed to set and incubated at 37^{0}C. This supports the growth of microbes exponentially. After 24 hours of incubation, the plates were removed and the diameter of the zone of inhibitions of test and control samples were measured in millimeters. The experiment was carried out in triplicate and the average reading was taken. (Onocha et al., 2010).

Agar diffusion: surface plate method for fungi

Sterile sabouraud dextrose agar was prepared accordingly and aseptically poured into sterile petri-dishes in triplicate and allowed to solidify properly. Fungal strains (*A. niger, C. albicans, P. notatum* and *R. stolonifer*) maintained on nutrient agar slopes and preserved at 4-5^{o}C for the assay was spread on the surface of the agar. The same procedure described for antibacterial assay above was carried out. The prepared concentrations of the samples, positive and negative controls were introduced into drilled holes in petri-dishes which were then incubated at 28^{o}C for 72 hours. Zone of inhibition of test and control samples were measured (Onocha et al., 2010).

Results

Phytochemical screening

The methanol extract (MST- 73.34% yield), the ethyl acetate (EST -5.95% yield) and hexane extract (HST -29.29% yield) were obtained as dark brown viscous substances. Phytochemical screening revealed the presence of secondary metabolites shown in Table 1.

Table 1: Result of Phytochemical Screening*

Phytochemicals	HST	EST	MST
Saponin	-	-	+
Flavonoids	-	-	-
Tannins	-	-	+
Steriods	+	-	-
Alkaloids	-	+	+
Reducing Sugar	-	-	-
Phenols	+	+	-
Cardiac Glycoside	+	+	+
Resins	-	-	+
Anthraquinones	-	-	+

*+ = Positive, - = Negative

Hydroxyl radical scavenging activity

The Absorbance at 285 nm of MST, EST, HST, ascorbic acid, butylated hydroxyl anisole (BHA) and α-tocopherol against the H_2O_2 radicals are shown in Table 2 while the percentage inhibition is shown in Figure 1.

The concentration of the samples as well as those for the standards that would be required to scanvenge 50 % of the free peroxide radicals (IC_{50}) were calculated from the equations of the line graphs and the results are as shown in Table 3

Table 2: Absorbance values of MST, EST, HST extracts and standards on H_2O_2 at 285 nm

CONC.(mg/ml)	MST	EST	HST	Ascorbic Acid	BHA	α-Tocopherol
1.0	2.046±0.040	2.304±0.004	2.046±0.040	0.689±0.002	2.257±0.026	2.951±0.041
0.5	1.240±0.001	1.491±0.009	1.240±0.001	0.356±0.003	1.975±0.003	2.874±0.064
0.25	0.722±0.001	0.846±0.005	0.722±0.001	0.138±0.001	1.770±0.017	2.251±0.022
0.125	0.433±0.001	0.527±0.003	0.433±0.001	0.191±0.001	1.731±0.008	1.781±0.002
0.0625	0.316±0.005	0.338±0.001	0.316±0.005	0.113±0.002	1.699±0.030	0.935±0.002

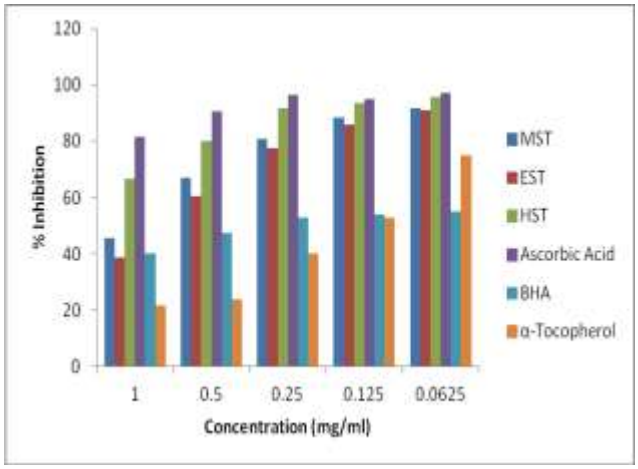

Figure 1: Percentage inhibition of *Sterculiar tragacantha* Lindl extracts and standards on H_2O_2

Table 3: The IC_{50} of the extracts and the standards used

SAMPLE	IC_{50} (mg/ml)
MST	0.890
EST	0.760
HST	1.510
Ascobic acid	2.976
BHA	0.385
α-tocopherol	0.236

Brine Shrimp toxicity Test

Toxicity to lower organism was measured using the Brine shrimp toxicity test. The number of survivals and the number of dead larvae were enumerated and the result is as presented in Table 4. From this data, the percentage LC_{50} value was determined using Probit analysis for each concentration. Report has it that secondary metabolites from plants which are active medicinally are most times toxic to Brine shrimp larvae *Artermia salina* nauplii which is a living organism with no advance nervous system (Aiyelaagbe, *et al*, 2009; Oloyede *et al*, 2010).

Table 4: Result of Brine Shrimp toxicity Test*

S/N	Plant Extract	Solvent	Conc. (µg/ml)	Control *N0*/ALV	*N0*	*ALV*	*NOD*	LC_{50}(µg/ml)	*G*
1	*HST*	Sea water and DMSO	1000	30	30	22	8		
			100	30	30	27	3	5869.6980	0.5120
			10	30	30	30	-		
2	*EST*	Sea water and DMSO	1000	30	30	10	20		
			100	30	30	25	5	487.0259	0.1745
			10	30	30	29	1		
3	*MST*	Sea water and DMSO	1000	30	30	11	19		
			100	30	30	23	7	482.8018	0.1574
			10	30	30	30	-		

**'N0' is* initial number of living larvae put into every concentration at the test start. *'ALV' is* the number of survival. *'NOD' is* the number of dead. *'LC_{50}' is* lethality concentration 50%. *G* is the degree of freedom. *MST*: Methanol extract of *S. tragacantha*, *EST:* Ethylacetate extract of *Sterculiar tragacantha*, *HST*: Hexane extract of *S. tragacantha*

Antimicrobial Assay of the Extracts

Zones of inhibition measured in millimeters were reported as seen in Table 5.

Discussion

Phytochemical screening of extract of *S. tragacantha* Lindl stem bark, showed the presence of steroids, phenolics and cardiac glycoside in the hexane extract; the presence of alkaloid, phenolics and cardiac glycosides in the ethyl acetate extract while saponin, tannin, alkaloid, cardiac glycoside, resins and anthraquinones were present in the methanol extract.

The ability of the extracts to scavenge hydroxyl radical was determined according to Oloyede et al., 2010; Onocha et al.,2011. In the hydrogen peroxide scavenging radical method, the percentage inhibition of the methanol extract was in the range of 45.71 - 91.625 %. The lowest concentration (0.0625 mg/ml) showed the highest percentage inhibition (91.625 %) (Figure 1).

The ethylacetate extract also showed similar trend (38.88 % at 1.0 mg/ml, 60.43 %, 77.55%, 86.01 % at 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml respectively, and 91.04 % at 0.0625 mg/ml). The hexane extract showed 66.71% at 1 mg/ml and 80.10%, 91.85%, 93.50% at 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml respectively and 95.63% at 0.0625 mg/ml. At the lowest concentration of 0.0625 mg/ml the percentage inhibition was the highest for all the extracts (Table 2 and Figure 1). This can be attributed to the presence as phenols and cardiac glycosides in the extracts.

The IC_{50} (the concentration of the samples required to scavenge 50 % of the hydroxyl radicals) was used to examine the antioxidant effectiveness of sample. The lower the IC_{50}, the greater the overall effectiveness of the suspected antioxidant sample. From the results obtained, it was revealed that the natural standard antioxidant, α-tocopherol showed the best antioxidant effectiveness with IC_{50} of 0.236 mg/ml followed by the synthetic antioxidant standard butylated hydroxyl anisole (*BHA*) as it had IC_{50} of 0.385 mg/ml. However, the test samples *EST*, *MST* and *HST* had IC_{50} of

Table 5: Antimicrobial activity of *MST, HST AND EST**

Conc.(mg/ml)	*Sa*	*Ec*	*B.Sab*	*Ps.a*	*Sal*	*Klebs*	*C.a*	*A.n*	*Phij*	*Pen*
200	30	26	24	26	24	26	20	18	16	18
100	26	20	20	20	18	18	18	16	14	16
50	24	18	16	18	16	16	16	14	12	12
25	16	14	14	14	14	12	14	10	10	10
12.5	14	12	12	12	12	19	10	-	-	-
6.25	12	10	10	10	10	-	-	-	-	-
			HST							
200	24	20	18	20	18	18	16	16	14	16
100	20	16	16	16	16	16	14	14	12	14
50	18	14	14	14	12	14	10	12	10	10
25	14	12	12	10	10	10	-	10	-	-
12.5	10	10	-	-	-	-	-	-	-	-
6.25	-	-	-	-	-	-	-	-	-	-
			EST							
200	20	16	18	16	14	16	14	14	14	12
100	18	14	14	14	12	14	12	12	10	10
50	14	12	12	10	10	12	10	10	-	-
25	10	10	10	-	-	10	-	-	-	-
12.5	-	-	-	-	-	-	-	-	-	-
6.25	-	-	-	-	-	-	-	-	-	-
-ve	—	—	—	—	—	—	—	—	—	—
+ve	38	38	40	38	38	38	28	28	26	28

S.a*: *Staphylococcus aureus.* ***E.c: Esherichia coli*, ***B.sab**: Bacillus subtilis*, ***Ps. a**: Pseudomonas aerugunosa.* ***Sal. t**: Salmonella typhi*, ***Klebs**: Klebsiellae pneumonia*, ***C. a**: Candida albicans*, ***A. n** : Aspergillus niger*, ***Pen** : Penicillum notatum*, ***Rhi**. : Rhizopus stolonifer* ***MST*** : Methanol extract of *S. tragacantha*, ***EST*** : Ethylacetate extract of *Sterculiar tragacantha*, ***HST*** : Hexane extract of *S. tragacantha*, -ve: Negative control; Methanol for methanolic extract, ethylacetate for ethylacetate extract and hexane for hexane extract. +ve: Positive control; Gentamicin 10 µg/ml for bacteria and Tioconazole 70% for fungi.

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0.760, 0.890 and 1.510 mg/ml respectively while the ascorbic acid standard had 2.977 mg/ml (Table 3). Therefore, the three test samples were more effective antioxidants compared to ascorbic acid but not as effective as α-tocopherol and butylated hydroxyl anisole. The order of decreasing antioxidant effectiveness is α- Tocopherol > *BHA* > *EST* > *MST* > *HST* > Ascorbic acid.

The brine shrimp lethality bioassay was conducted to assess the *in vitro* toxicity effect of the plant extracts. The guiding principle for the brine shrimp toxicity test is: $Lc_{50} \leq 1000$ is considered toxic while $Lc_{50} \geq 1000$ is considered not toxic. The LC_{50} values of the methanol and ethyl acetate extract are 482.8018 µg/ml and 487.0259 µg/ml respectively indicating that the extracts were toxic while hexane extract with LC_{50} of 5869.6980 µg/ml was non- toxic (Table 4).

The antimicrobial assay of the three extracts showed that at very high concentration of 200 mg/ml and 100 mg/ml, the three extracts were active against the ten micro-organisms used. At 200 mg/ml, the range was 30 mm to 16 mm while at 100 mg/ml, the range was 26 mm to 10 mm zone of inhibition. However the methanol, ethylacetate and hexane extracts were most active against *Staphylococcus aureus.*
At lower concentration of 50 mg/ml and 25 mg/ml, the methanol extract was active against all the organisms (Table 5). At 12.5 mg/ml, the methanol extract was found to be inactive against *Aspergilum niger, Penicillum notatum* and *Phijupus stolaniter* and at the lowest concentration of 6.25 mg/ml, the methanolic extract was active against some of the organisms, though it was less prominent compared to the activities at higher concentration; *Klebsiellae pneumonia, Candida abicans, Aspergillus niger, Penicillum notatum* and *Phijupus stolaniter* were resistant to all the extract at a concentration of 6.25 mg/ml (Table 5).
The hexane extract at 50 mg/ml was active against all the organisms in the range of 14 mm to 10 mm zone of inhibition, with *Phijupus stoloniter* and *Penicillum notatum* showing higher resistance.
The ethyl acetate extract at 50 mg/ml was active against all the test organisms except *Penicillum notatum* and *Phijupus stoloniter*. At 25 mg/ml, the extract showed low activity against *Staphylococcus aureeus, Esherichia coli, Bacillus subtilis and Klebsiellae pueumonia* (10 mm zone of inhibition) and inactive against the others, while at lower concentration of 12.5 mg/ml and 6.25 mg/ml, ethyl acetate extract showed no activity (Table 5).

Conclusion

Secondary metabolites; saponin, tannins, alkaloids, phenols, cardiac glycosides, resins and antraquinones were found in hexane, ethylacetate and methanol extract of *Sterculiar trangacantha* Lndl. The hydroxyl radical scavenging assay revealed that the percentage inhibition for all the extract was above 90 %. The ethylacetate and methanol extract were toxic with LC_{50} value of 487.03 µg/ml and 482.80 µg/ml respectively, while the hexane is non toxic with $LC_{50} \geq 1000$ µg/ml.

The ability of the extracts to scavenge hydroxyl radicals and inhibit growth of microorganisms makes the plant a potential source of antioxidant and antimicrobial compounds and also validates the ethno medicinal use of *Sterculiar trangacantha* Lndl.

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Current Research Interest

Chemistry of Natural Products and their Medicinal Uses.
Phytochemical evaluation of Medicinal Plants with emphasies on antioxidant, antimicrobial, anticancer and hepatotoxic activities.
Isolation and Characterisation of bioactive chemical compounds from plants
Synthesis of Schiff and Mannich Bases and invesitigation of their biology activities.

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