Pharmacognostic studies and antioxidant activity of the stem-bark of *Entandrophragma utile* Dawe & Sprague (Meliaceae)

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ABSTRACT

The stem-bark of *Entandrophragma utile* (Meliaceae) is used traditionally for treatment of rheumatism, eye inflammation, sickle cell disease, gastric and duodenal ulcers in Nigeria. The aim of this study is to evaluate the pharmacognostic and antioxidant properties of the stem-bark of *E. utile*. Macroscopic and microscopic examinations, physicochemical parameters as well as phytochemical analyses of the powdered stem bark are performed. The antioxidant activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effects. The macroscopic studies indicated that the stem-bark is brown, bitter, rough, channeled or squilled and has a smooth fracture type. The microscopy showed the presence of bundles of fibres, single fibres, cork cells, and sclereids. Chemo-microscopy showed the presence of lignified cell walls, suberized/cuticular cell walls, tannins and starch. Physicochemical constants are: moisture-content 8.3 ± 0.03 %, alcohol-soluble extractive 0.8 ± 0.07 % and 0.4 ± 0.03 % water-soluble extractive values. The phytochemical screening showed the presence of tannin, saponin and flavonoid. The total phenolic and flavonoid contents of the extract was found to be 452.8±39.4 (GAE/g of dried extract mg/g) and 15,673±261.7 (QE mg/g) respectively. The IC_{50} was found to be 0.75 mg/mL. The study showed that the stem bark extract is a good source of polyphenols that could be responsible for the observed antioxidant activity of the plant.

Keywords: *Entandrophragma utile*, Pharmacognostic studies, Antioxidant, Chemo-microscopy, Stem-bark**.**

INTRODUCTION

Herbal medicine also called botanical medicine or phyto-medicine has a long tradition of use outside conventional medicine. It is becoming more main stream as improvements in analysis and quality control, along with advances in clinical research, show the value of herbal medicine in treating and preventing diseases[1].

Herbal medicines as the major remedy in traditional medical system have been used in medical practice for thousands of years and have made a great contribution to maintain human health[2].

The advantages of herbal medicines are their ready availability, inexpensiveness and less or no side effects but they are easily adulterated[3].

According to Cragg and Newman[4], the utility of natural products as sources of novel structures is still feasible. Up to 50% of the approved drugs during the last 30 years are obtained directly or indirectly from natural products.

Pharmacognostic study is the preliminary step in the standardization of crude drugs[5]. It gives the scientific information regarding the purity and quality of plant drugs[6]. The detailed pharmacognostic evaluation gives valuable information regarding the morphology, microscopic and physical characteristics of the crude drugs[7].

Adulteration or substitution is a common concern in the use of herbal medicines. It is the replacement of original plant with another plant material or intentionally adding any foreign substance to increase the weight or potency of the product or to decrease its cost[3]. Therapeutic efficacy of medicinal plants

depends upon the quality and quantity of chemical constituents. The misuse of herbal medicine or natural products starts with wrong identification. This problem can be solved by pharmacognostic studies of medicinal plants.

E. utile is widespread, occurring from Sierra Leone to Uganda, DR Congo and Angola. It is commonly known as Sipo mahogany or African cedar. Its wild population is greatly diminished[8]. It is also found in Nigeria and has wide medicinal applications.

The bark is used traditionally to treat malaria and heal peptic ulcers[9,10]. Sap from the plant's bark when taken internally is used to treat stomach ache and kidney pain[11]. When applied into affected joints, it relieves rheumatism, eye inflammations and treats otitis. The bark extracts have shown fungicidal activity against *Pyriculana oryzae*[11].

Various activities of parts of the plant have been investigated and reported. These include gastric protective property[10] and antisickling activity[12].

This study aimed to evaluate the pharmacognostic properties of the stem-bark of *E. utile* with the hope of aiding its standardization, quality control of its products and assessing its anti-oxidant capacity (DPPH).

MATERIALS AND METHODS

Collection and identification of plant material

The stem-bark of *E. utile* was collected at Oloke-meje area, Oyo State, Nigeria in March, 2017. The plant was identified by Mr Bolu-Ajayi and authenticated at the Herbarium unit of the Department of Plant Biology, University of Ilorin, Nigeria where a voucher specimen (UILH/001/1325) was deposited.

Methods

Preparation and extraction of plant material

The stem-bark was carefully cleaned to remove dust and sand particles and chopped into pieces. They were air-dried on the bench top and further dried in the oven at 40 ºC for three weeks. It was then ground in a mechanical grinder and thereafter stored in a tightly covered amber coloured glass jar.

The fresh stem-bark was used for the macroscopic examination, whereas the powdered stem-bark was used for the microscopy, physicochemical and phytochemical analyses.

Macroscopic Examination

The fresh stem-bark of *E. utile* was examined for colour, odour, taste, shape, surface characteristics, texture and fracture type according to the methods of Evans [13].

Microscopic Examination

The powdered stem-bark was evaluated for fibres, phloem, calcium oxalate crystals, sclereids and cork cells. This was done by adding few drops of chloral hydrate solution (clearing reagent) to the powdered plant material on a slide, covered with a cover slip and heated gently over a bunsen burner. After clearing, a drop of glycerol was added to the sample, mounted on the microscope and examined for the different microscopic features mentioned above. Photographs of selected diagnostic features were taken using a camera.

Chemo-microscopy

Chemo-microscopic examinations were carried out on the powdered drug. Various cell wall materials such as lignin, hemicellulose and cell contents such as starch, oils, tannins and calcium oxalate crystals were observed for, following methods described by Elufioye and Olaifa[14].

Test for lignin

To a small amount of the cleared powdered plant material, phloroglucinol and concentrated HCL were added and viewed under the microscope; appearance of pink to cherry red coloration will indicate lignified cell walls.

Test for suberized or cuticular cell wall

To a small amount of the cleared powdered plant material, 1-2 drops of Sudan red was added followed by gentle warming and viewing under the microscope; suberized or cuticular cell walls are stained orange-red or red.

Test for tannins

To a small amount of the cleared powdered plant material, 5% $FeCl_3$ solution was added and viewed under the microscope; tannins turn bluish black or greenish black.

Test for calcium oxalate crystals

Calcium oxalate crystals are seen as bright structures of definite shapes and sizes in cleared powdered plant material. On addition of 80% H_2SO_4 and viewing under microscope, disappearance of calcium oxalate crystals confirms their presence.

Test for calcium carbonate

Small amount of the cleared powdered plant material was mounted on a slide and irrigated with acetic acid through the side of the cover slip. Dissolution of the crystals with effervescence indicates the presence of calcium carbonates.

Determination of physicochemical constants

Physicochemical parameters such as moisture content, alcohol and water soluble extractive values were studied using the method described by Karumari *et al*[15]and Elufioye and Olaifa[14].

Determination of moisture content: The moisture content was determined by loss on drying method. 2 g of powdered stem-bark was measured accurately and placed into a clean evaporating dish of known weight. The dish was then transferred into a pre-heated oven set at 105^{o}C. After one hour, the weight of the powered drug and evaporating dish was determined and returned into the oven. The weighing was done repeatedly at 30 mins interval until a final constant weight was achieved. The moisture content was calculated with reference to the original weight of the powdered drug sample as follows;

$$\%\ \text{Moisture content} = \frac{\text{Final weight of sample}}{\text{Original weight of the sample}} \text{X } 100$$

Determination of alcohol-soluble extractive value: 5 g of powdered *E. utile* stem-bark was accurately weighed into 250 mL stoppered conical flask. 100 mL of 90% ethanol was added and tightly closed. The flask was kept aside for 24 hours with frequent shaking for the first 6 hours and then allowed to stand for 18 hours, after which it was filtered rapidly to avoid loss of solvent. 25 mL of the filtrate was collected, transferred to a weighed porcelain dish, evaporated to dryness on a water bath and then dried in an oven at 90^{o}C to constant weight and the final weight determined. The alcohol-soluble extractive value was calculated using the formula:

$$\%\ \text{Alcohol} - \text{soluble extractive value} = \frac{\text{Final weight of residue in 25 mL}}{\text{25 ml}} \text{X } 100$$

Determination of Water-soluble extractive value: The same procedure above was repeated for determination of water-soluble extractive value but water was used as the extracting solvent instead of 90% ethanol.

Phytochemical investigations

Qualitative analysis

Various phytochemical investigations were performed on the ethanol extract of *E. utile* following standard procedures as described by Kokate[16]. Colour intensity was used to categorize the presence of each phytochemical into copious, moderate or slight (trace).

Test for Flavonoids

To a test tube containing 1 mL of extract, a few drops of dilute Sodium hydroxide (NaOH) solution was added. An intense yellow colour produced in the extract which becomes colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

Test for Anthraquinones (Borntrager's reaction)

To 2 mL of chloroform extract, dilute (10%) ammonia solution was added. A pink-red colour in the ammoniacal (lower) layer indicates the presence of anthraquinones.

Test for Tannins

To a test tube containing the extract, 1 mL of 5% Ferric chloride was added. The presence of tannin is indicated by the formation of bluish black or greenish black precipitate.

Test for Saponins

The extract was diluted with 20 mL distilled water and was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam indicates the presence of saponin.

Test for Cardiac glycosides
To a test tube containing 5 mL of extract was added 2 mL of glacial Acetic acid containing a drop of Ferric chloride ($FeCl_3$) solution. It was then underplayed with 1 mL concentrated sulphuric acid (H_2SO_4). A brown ring at the interface indicates a de-oxy sugar characteristic of cardenolides.

Test for Steroids
The extract (1 mL) was dissolved in 10 mL chloroform and equal volume of concentrated sulphuric acid (H_2SO_4) was added down the side of the test tube. The upper layer turns red and sulphuric acid layer show yellow with green fluorescent. This indicates the presence of steroids.

Test for Terpenoids
To a test tube containing 5 mL of extract was mixed with 2 mL Chloroform. 3 mL of concentrated sulphuric acid (H_2SO_4) was then added to form a layer. Formation of a reddish brown precipitate colouration at the interface indicates the presence of terpenoids.

Test for Alkaloids
Few drops of Mayer's reagent, Hager's reagent and Wagner's reagent were separately added to the extract. Alkaloids solution produces cream coloured precipitate, prominent yellow precipitate and reddish brown precipitate respectively.

Quantitative analysis

Determination of Total Phenolic Content

The total phenolic content (TPC) of the extract was determined by modified Folin-Ciocalteu reagent method[17]. Gallic acid was used as a standard. Concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 µg/mL of Gallic acid and 0.28mg/mL of the extract were prepared in methanol. 1.0 mL of each preparation was mixed with 1 mL Folin Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and vortexed. After 5mins, 0.8 mL (75 g/L) of sodium carbonate was then added, vortexed for 15 seconds and allowed to stand for 30 minutes at room temperature. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. All determinations were performed in triplicates. The TPC was expressed as mg/g Gallic acid equivalent using the following equation based on the calibration curve: y = 1.632x, where y is the absorbance, x is the concentration.

Determination of Total Flavonoid Content
Total flavonoid was estimated using the method of Miliauskas *et al* [18]. Concentrations of 0.3 mg/mL of the extract in methanol was prepared, while Quercetin concentrations of 0.03, 0.06, 0.09, 0.12 and 0.15 mg/ mL also prepared in methanol were used to obtain the calibration curve. 2.0 mL of 2% Aluminium Chloride ($AlCl_3$) in ethanol was added to 2.0 mL of each preparation and allowed to stand at room temperature for 60 minutes. The absorbance was measured at 420 nm after 60 minutes. The estimation of total flavonoids content in the crude extract was carried out in triplicate and the result averaged. The total flavonoid content was calculated as Quercetin equivalent (mg/g) using the equation based on the calibration curve: y=0.2267x, where y is the absorbance and x is the concentration incorporated into the following formula:

$$\mathbf{T_{(QE)}} = \frac{\mathbf{(C\ x\ V)}}{\mathbf{M}}$$

Where
T= Total Flavonoid content (Quercetin equivalent), mg/g plant extract
C = Concentration of Quercetin from standard curve mg/mL
V = Volume of extract used during the assay (mL)
M = Mass of extract used during assay (g)

Antioxidant Assay

Determination of DPPH radical scavenging activity

This was done using DPPH radical-scavenging activity on the test extract as described by Deng *et al* [19]. Different concentrations (0.02 – 5.0 µg/mL) were prepared. Equal volume of the extract was added each with equal volume of DPPH (100 µM). The mixture was kept in the dark at room temperature for 30 minutes. Ascorbic acid was used as standard control. Three replicates were made for each test sample. The absorbance (A) was then measured at 518 nm after 30 minutes and converted into the percentage DPPH

antioxidant activity using the following equation:

$$\% \text{ DPPH} = \left(\frac{\text{Absorbance (DPPH)} - \text{Absorbance (Extract)}}{\text{Absorbance (DPPH)}}\right) x \frac{100}{1}$$

The IC_{50} value which denotes the concentration of sample required to scavenge 50% of DPPH free radicals was determined.

Statistical Analysis

The results were expressed as Mean ± Standard Error of Mean (SEM). Statistical analysis of the data was done using descriptive statistical tools, with the aid of Graph Pad Prism 6. Line chart was used to express the obtained data.

RESULTS AND DISCUSSION

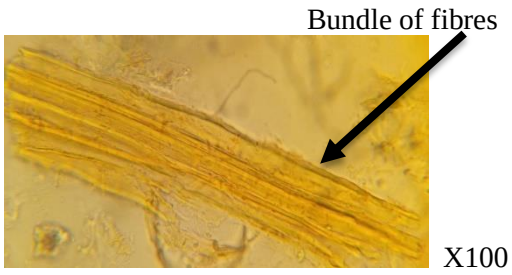
Results

Macroscopic Examination of *E. utile* Stem-bark

The stem-bark is channeled in shape, rough and with a smooth fracture type. Outer and inner surface is brownish in colour, with characteristic odour and bitter taste.

Microscopic Examination

The microscopic examination shows the presence of bundles of fibres, cork cells, lignified sclereid, and single fibre (Fig. 1).



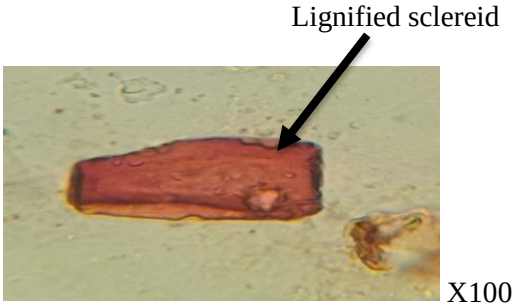


Fig. 1: Microscopic features of powdered stem-bark of *E. utile*

Chemo-microscopic Evaluation and Determination of Physicochemical values

The chemo-microscopic and physicochemical parameters are unique to a plant and are important in correct identification of the plant. Chemo-microscopic evaluation of *E. utile* revealed the presence of cellulose, lignins, tannins, oils, starch grains and protein as shown in table 1.

Table 1: Chemo-microscopic evaluation of *E. utile*

Parameter	Result
Cellulose	+
Lignin	+
Tannins	+
Starch	+
Oils	+
Calcium carbonate	+
Calcium oxalate crystals	-
Protein	+

Alkaloids	-

[+] = Present - = Absent

Physical constants were also established for the plant (table 2).

Table 2: Physicochemical values of *E. utile* stem-bark

Parameter	Percentage
Moisture content	8.35±0.03%
Water extractive	0.4±0.03%
Alcohol extractive	0.8±0.07%

Phytochemical Evaluation of *E. utile* Stem Bark

Qualitative analysis

Results of phytochemical screening with the ethanol extract of the plant was observed to test positive to secondary metabolites like flavonoids, terpenoids, tannins, and saponins (Table 3).

Table 3: Preliminary phytochemical screening of *E. utile*

Constituents	Test	Observation	Inference
Alkaloids	Mayer's reagent	No creamy white precipitate	-
	Hager's reagent	No yellow precipitate	-
	Wagner's reagent	No reddish brown precipitate	-
Anthraquinones	Borntrager's	No pink colour	-
Cardiac glycosides	Keller-killiani	No reddish brown at interphase	-
Flavonoids	NaOH test	Yellow colouration turns colourless with HCl	++
Saponins	Frothing test	Persistent froth	+
Terpenoids	Salkowski's test	Brown colour at interphase	-
Steroids	Liebermann-Burchard	Greenish colouration	-
Tannins	$FeCl_3$ test	Greenish black precipitate	+++

+++ (copiously present), ++ (moderately present), + (slightly present), - (absent)

Quantitative analysis

E. utile extract had 452.8±39.4 (GAE/g of dried extract mg/g) and 15,673±261.73 (QE mg/g) total phenolic[1] and flavonoid[2] content respectively.

[1]: mg/g Gallic Acid Equivalent

[2]: mg/g Quercetin Equivalent

Values were performed in triplicates and represented as mean ± SEM

DPPH radical scavenging activity

The radical scavenging effect of the extract and ascorbic acid on DPPH concentration decreased in the order: ascorbic acid > *E. utile* extract with IC_{50} values of 0.05 and 0.75 mg/ml respectively.

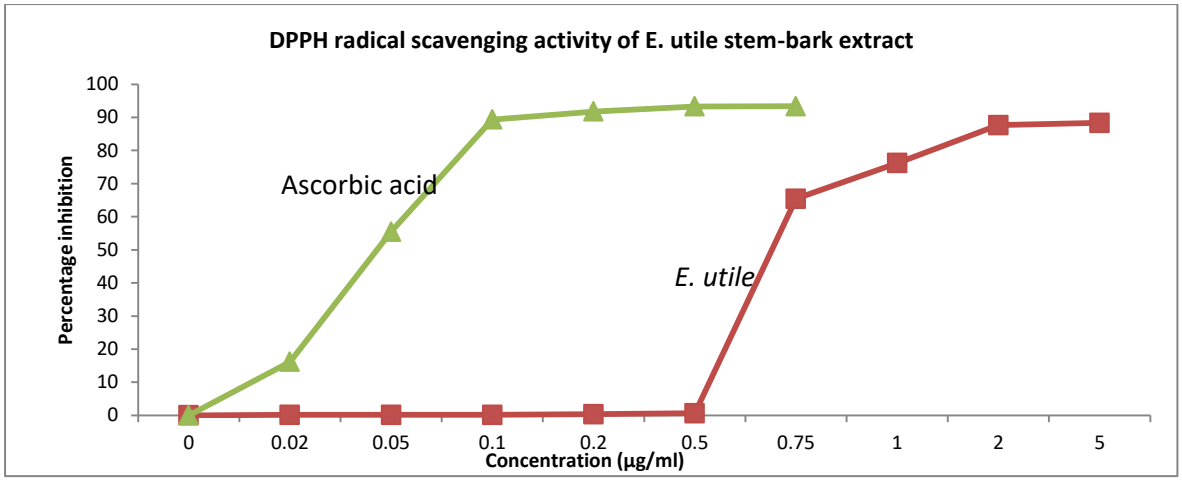


Fig. 2: DPPH radical scavenging activity of *E. utile* stem-bark extract. Values are means of 3 replicates.

DISCUSSION

Microscopic and chemo-microscopic evaluation guides the determination of several diagnostic characters, cell wall material and cell inclusion which aid in the botanical identification, authentication and standardization of plant materials[20]. The determination of physicochemical parameters is significant in the assessment of adulterants and improper handling of crude drugs[21]. Pharmacognostic and phytochemical studies of medicinal plants is important in other to ensure reproducible quality of herbal medicines which contributes to their efficacy and safety[21]. The presence of bioactive secondary metabolites such as terpenoids, saponins, flavonoids, alkaloids, phenols and tannins, are responsible primarily for observed pharmacological potencies of medicinal plants
The macroscopic examination of the stem bark of *E. utile* showed a bark that is brown with characteristic odour, bitter taste, texture is rough, fracture type is smooth and the shape is channeled and squiled. The microscopic and chemo-microscopic evaluations of stem bark were carried out. Several diagnostic characters including, cuticular cell wall, fibres, cork cells and tannins that can aid in the botanical identification, authentication and standardization of the plant material were identified.

Extractive values are useful indicators of the approximate amount of the chemical constituents in a crude drug and also help in the estimation of specific constituents soluble in a particular solvent. The moisture content of *E. utile* was 8.35±0.03%. This value is high, hence care must be taken during storage of the crude drug to maintain stability and avoid microbial contamination and deterioration[22].
The phytochemical analysis shows the presence of secondary metabolites like saponins, flavonoids, phenols, tannins while alkaloids and phytosterols are absent.
Antioxidants are compounds that function as free radical scavengers, reducing agents and quenchers of singlet-oxygen formation. They are regarded as protection agents minimizing reactive oxygen species related oxidative damage in the body and reducing chronic diseases[23]. Polyphenolic compounds of plants especially flavonoids and phenols have redox properties, which allow them to act as antioxidants. Their free radical scavenging ability is facilitated by their hydroxyl groups[24]. Flavonoids and phenol are used for the prevention and cure of various ailments which are associated with free radicals. Hence, the total phenolic and flavonoid concentration can be used as a basis for rapid screening of antioxidant activity[24]. The extraction and quantification of these phenolic compounds in medicinal plants are therefore necessary to allow assessment and eventual value added utilization[25]. *E. utile* extract had 452.8±39.4 (GAE/g of dried extract mg/g) and 15,673±261.73 (QE mg/g) total phenolic and flavonoid content respectively.
DPPH is a stable free radical compound and its radical-scavenging assay evaluates the ability of a compound or an extract to scavenge free radicals and to promote the formation of the non-radical form DPPH-H by hydrogen-donating action[26]. Potential source of natural antioxidants are plants which act as reducing

agents, hydrogen donators, oxidant and free radical scavengers. The antioxidant potentials of plant extracts can be expressed on the basis of their IC_{50} values. The lower the IC_{50} value, the more potent is the extract as antioxidant or a free radical scavenger[27]. It can be deduced from the result (Fig. 2) that a decrease in DPPH concentration was observed due to the scavenging power of the extract and ascorbic acid. The radical scavenging effect of the extract and ascorbic acid on DPPH concentration decreased in the order: ascorbic acid > *E. utile* extract with IC_{50} values of 0.05 and 0.75 mg/ml, respectively, which suggests that *E. utile* stem-bark extract possess hydrogen donating capabilities and thus, scavenging of the free radicals.

The DPPH radical scavenging activity is also a measure of non-enzymatic antioxidant activity of compounds[19]. Therefore, the stem-bark extract of *E. utile* could stimulate antioxidant enzymes through activation of estrogen receptor/PI3-kinase-dependent pathway[28].

The polyphenolic constituents present in the extract could be responsible for the observed antioxidant activity in this study.

CONCLUSION

The study provides macroscopic and microscopic features of the stem-bark. The phytochemical studies aids in providing valuable information about the chemical composition of the plant material and showed the presence of saponins, phenols, tannins and flavonoids in the stem-bark of *E. utile*.

It also provides pharmacognostic data, which could be used to determine correct identity and purity of stem-bark of the plant *E. utile* and for the detection of adulteration. *E. utile* possess antioxidant properties with DPPH radical scavenging effect and presence of phenolic compound which could be a potential source of natural antioxidant useful in the management of free radical mediated diseases.

RECOMMENDATIONS

Further studies should be carried out to isolate active principles present in the plant which are responsible for the acclaimed medicinal properties.

There is an urgent need for conservation and research on this plant before it leads to extinction.

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