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Phytochemical screening and antioxidant activity of the stem bark extracts of *Diospyros mespiliformis*: a medicinal plant in Bauchi

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Abstract
Approximately, about 80% of rural populations in developing countries rely on traditional medicinal plants for their health care needs. As a result, people have developed their knowledge of these traditional medicines through their experiences and daily observations. In Bauchi, this information is deeply rooted in their culture, transferred from one generation to the next, orally and along gender lines. *Diospyros mespiliformis*, commonly known as African ebony is a large deciduous tree belonging to the family Ebanaceae, it has a wider range of Ethnomedicinal uses, most of which is yet to be substantiated scientifically. Traditionally, it's used as an astringent, febrifuge, hemostatic, mild laxative, stimulant, vermifuge and to facilitate child birth. This study was aimed to determine the phytochemical constituents and antioxidant activity of the stem bark extracts of *D. mespilifomis*. The stem bark was collected within Bauchi Local Government Area, Bauchi State, identified by a botanist, Department of Biological Sciences, ATBU, Bauchi, air dried and ground into fine powder. The fine powder (100 g) was soaked in a 400 ml of 80% v/v methanol for about 48 hrs. The extraction yield was determined (12.50% w/w). The crude methanol extract was then partitioned sequentially in petroleum ether and ethylacetate. Standard method was used to screen for the phytochemicals and DPPH radical scavenging assay was employed to determine the antioxidant activity of the solvents extract. The phytochemical screening revealed the presence of alkaloids, tannins, phenolics, flavonoids, saponins, and glycosides, in the crude methanol extracts, while saponins, glycosides, terpenoids and stroids were present in petroleum ether fraction and ethylacetate fraction was also shown to contains tannins, flavoinoids, phenolics and alkaloids. DPPH radical scavenging assay revealed that the ethylacetate extract has the highest antioxidant potential (IC_{50}= 30.69 ± 1.28), followed by methanol extract (IC_{50}= 32.83 ± 1.29) and petroleum ether extract has the least antioxidant potential (IC_{50} = 43.80 ± 1.83). However the activity of the extracts was found to be less when compared to the standard (Ascorbic acid), IC_{50} = 25.65 ± 0.80 µg/cm^3 at 20 µg/cm^3. The results of the study supports the claims of efficacy reported in folk uses of the stem bark of the plant in the treatment of disease caused by free radicals and if further purified can be used to source novel antioxidants.

Keywords: *Diospyros mespiliformis*, antioxidant activity, DPPH, phytochemical screening, ethnomedicines, ebanaceae

1. Introduction
Traditional medicine as defined by World Health Organization (WHO, 2008) [24] may be summarized as the sum total of all the knowledge and practical, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. Traditional medicine might also be considered as a solid amalgamation of dynamic medical know-how and ancestral experience (Mornier, 2016) [13].
Medicinal plants are the richest bio-resource of drugs in traditional systems of medicines, modern medicines, pharmaceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Tiwari *et al.* 2011) [22]. Plant materials have remained central to tradomedical practices and have useful sources of new drugs. Although Orthodox medical practice is generally acceptable, alternative healthcare is still relied on all over in the world (Ozulua & Alonge, 2008) [16]. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decades, with more intensive studies for natural therapies. The use of compounds for pharmacological properties has gradually increased in different parts of the world.
According to (Audu, 1995) [5], about 85% of the populations of Bauchi State, Nigeria, were rural dwellers; and they use herbal treatment for their medication. The use of herbal drugs in treatment of diseases is found among all sections of people in Bauchi. The herbs are found in open spaces, shops and in the market sold by traditional practitioners. There are others also that move about with the herbs in their vehicles and advertise through loud speakers. From time immemorial, medicinal plants have been found to be important therapeutic aid for various ailments and diseases. Almost all cultures have depended either partially or fully on herbal medicine because of its availability, efficacy, affordability, low toxicity and acceptability (Coke, & Ogundele, 2002) [6]. Phytochemicals are non-nutritive plant chemicals produced by plants to protect themselves against environmental stressors like cold, heat, bacteria, fungi, etc, (Ahmad, & Urooj, 2010) [2]. They have curative and prophylactic properties against human and animal diseases (Argal, & Pathak, 2006) [4]. There are many phytochemicals in fruits, leaves, barks and roots of plants. Each of these phytochemicals may cure or prevent a disease singly or in synergism with some others. (Poongothoi, 2011) [19]
An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation and reduction of molecules are

essential to life; they represent normal phenomena that occur in cell metabolism. Among substances involved in oxidation-reduction reactions of molecules are free radicals, which are organic or inorganic compounds having one or more unpaired electrons on their valence shell, they are chemically unstable and very reactive (Lushchak, 2014) [11]. In organisms, reactive oxygen species (ROS) and reactive nitrogen species are involved in metabolic processes such as energy production, regulation of cell growth, intercellular signaling, phagocytosis and synthesis of important biological molecules (Lushchak, 2014) [11]. For many years, chemists have known that free radicals instigate oxidation, which can be controlled by a range of antioxidants substances. Antioxidants exert their activity by scavenging the free radicals, thereby giving rise to a fairly stable radical. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals if not scavenged effectively in time, they may damage crucial bio molecules like lipids, proteins including those present in all membranes, mitochondria and the DNA resulting in abnormalities leading to diseases conditions (James, 2012) [9]. Thus, free radicals are involved in a number of diseases including: tumor inflammation, hemorrhagic shock, diabetes, infertility, asthma, cardiovascular disorder etc, among others (James, 2012) [9].

1.1 Description of the Plant under Study

Ebony tree (*Diospyros mespiliformis)*

Diospyros mespiliformis (Ebanaceae) is one of the most widely distributed African trees and it grows in Nigeria under wider range of conditions than any other, this specie is widespread north up to Sahara (National Research Council, 2008) [15]. Some native names of *D. mespiliformis* include; Hausa: Kanya, Yoruba: Igidudu, Fulani: Nelbi, Kanuri: Bergem, Arabic: Jukham. It is commonly called Jackal-berry or African ebony. It is found in Savannah and Northern low land forest. It is an ever green tree of 12-15 m height but sometimes reaching up to 20 m or more in the rain forest (Aliyu, 2006) [3]. The leaves are simple alternate dark green with small hairs on the underside of old leaves. The plant is diaciouss and flowers in the months of April and May. Mature fruits are large yellow berries (NRC, 2008). The fruit of this plant is a traditional food of high nutritive value in Africa. The leaf extract is used against fever and syphilis, as an antidote to variety of poisonous substances. It is also used as an anthelmintic, insecticide and as a wound dressing agent (NRC, 2008).

1.2 Taxonomical Classification of the Plant under Study

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliosida
Order	:	Ebanales
Family	:	Ebanaceae
Genus	:	Diospyros
Specie	:	D. *mespiliformis*

1.3 Ethnomedicinal Uses of the Plant under Study

Leaves: Used as astringent, febrifuge, hemostatic, mildly laxative, stimulant. and vermifuge. Infusion of the leaves is used in treatment of fevers, pneumonia, syphilis, leprosy and yaws (NRC, 2008). The leaves are also used for treatment of headache, arthritis and skin infections. The leaves and fruits are chewed or applied as infusion for treating gingivitis, toothache, and for wound dressing to prevent infection (Abba *et al.* 2016) [1].

Roots and Barks

An infusion of the bark is used to treat stomach ache. Bark and roots are also used for infections such as malaria, pneumonia, syphilis, leprosy, dermatomycoses, as an anthelmintic and to facilitate childbirth (Abba *et al.* 2016) [1]. Barks and roots are used as psycho-pharmacological drug and to treat tumor (Mohamed, 2009) [12]. Roasted and pulverized roots are taken to treat jaundice. Bark preparations are administered to treat cough, bronchial diseases, tuberculosis, syphilis and leprosy, and applied externally to wounds, ulcers, bruises and furuncles. The bark is also used in veterinary medicine as vermifuge (El-Kamali, 2011) [7].

Fruits and Seeds

Fruits decoction or infusions are taken to treat dysentery, diarrhea, and menorrhagia. Fruit ash is applied to fungal skin infections and fruit powder to ulcers, whereas seed decoctions are administered against headache. Twigs are chewed to clean teeth (El-Kamali, 2011) [7]. Its seeds are also known to have nutraceutical value in managing high cholesterol, reducing risk of type-2 diabetes, and for weight control **(**Preedy *et al.* 2011) [18].

2. Materials and Method

2.1 Materials

2.1.1 Apparatus/Equipments

UV-Visible Spectrophotometer (JENWAY 6302), Rotary shaker (6155 Large Orbital Shaker), Rotary evaporator (RE-201D), Whatmann filter paper No.1, weighing balance, separatory funnel, glass rod, test-tubes, test-tube rack, conical flasks and other necessary laboratory apparatus.

2.1.2 Reagents and Solvents

The reagents used for the extraction includes: methanol (JHD), petroleum ether (Flinn Scientific) and ethyl acetate (Pure Chems). Other chemicals used for the analysis are benzene, ammonia solution, ferric chloride, sulphuric acid, potassium iodide, acetic acid, hydrochloric acid, acetic anhydride, chloroform, distilled water, DPPH (Sigma Aldrich), and Ascorbic Acid (Quali-Tech).

2.1.3 Preparation of 0.10 mM DPPH Solution

10 mg (0.01 g) of DPPH powder was weighed and dissolved in a beaker containing small amount of absolute methanol and it was then quantitatively transferred into 100 ml volumetric flask and made up to volume with absolute methanol. The resulting DPPH solution has a concentration of 0.25 mM. From the stock DPPH solution, a 20.0 ml was then measured and transferred quantitatively into a 50 ml volumetric flask and made up to volume with absolute methanol. The resulting DPPH solution has a concentration of 0.10 mM.

2.1.4 Preparation 0.10 mg/ml Ascorbic Acid Solution

A 1000 mg (1.0 g) of Ascorbic acid was weighed and dissolved in a beaker containing small amount of distilled water and it was then quantitatively transferred into 100 ml volumetric flask and made up to volume with distilled water. The resulting Ascorbic acid solution has a

concentration of 10 mg/ml. From the stock solution, a 2.0 ml was measured and transferred quantitatively into a 50 ml volumetric flask and made up to volume with distilled water. The resulting Ascorbic acid solution has a concentration of 0.10 mg/ml (100 µg/ml). The prepared solution was diluted to various concentrations required for the analysis.

2.2 Collection and Identification of Samples

The fresh stem bark of the plant was collected from Bauchi Local Government Area of Bauchi state, and the identification of the plant was authenticated by a professional botanist in the Department of Biological Sciences, Faculty of Science, Abubakar Tafawa Balewa University, Bauchi.

2.3 Methods

2.3.1 Grinding and Extraction

The stem barks were washed thoroughly thrice with distilled water and dried under fan for seven (7) days. The fine powder was obtained from stem bark using laboratory mortar and pestle. About 100 g of stem bark dry powder was extracted with 400 ml of 80% v/v methanol solvent using rotary shaker. After completion of the extraction, the prepared extract was then filtered and concentrated using a rotary evaporator and dry in an oven at 40 ^{0}C to obtain a crude methanol fraction (CF). The extraction yield was determined (12.50% w/w). Partition of the CF was performed further by the method of Zhao *et al.* (2009) with slight modification as described by Parasad *et al.* (2009) [17]. The dried CF was then dissolved in 100 ml water and was then portioned sequentially with 50 ml each of petroleum ether and ethylacetate respectively 3 times each. The fraction of each solvent was then collected and concentrated using a rotary evaporator to remove the solvents.

2.4 Preparation of Extracts

2.4.1 Stock solution

A 1000 mg (1.0 g) of each extracts was weighed and dissolved in a beaker containing small amount of distilled water and it was then quantitatively transferred into 100 ml volumetric flask and made up to volume with distilled water. The resulting extracts solutions have a concentration of 10 mg/ml.

2.4.2 Serial Dilution of the Extracts

From the stock solution prepared above, a 2.0 ml was measured and transferred quantitatively into a 50 ml volumetric flask and made up to volume with distilled water. The resulting extracts solutions have a concentration of 0.10 mg/ml (100 µg/ml). The prepared solution was diluted to various concentrations required for the analysis.

2.5 Phytochemical Screening

The preliminary phytochemical analysis of the plants extracts was performed using standard procedures (Santhi and Sangottuvel, 2016) [21]. To detect the presences of bioactive components in the stem bark extracts of *Diospyros mespiliformis*.

2.5.1 Test for Phenolic Compounds

Ferric chloride test: The extracts were dissolved in about 10 cm^3 of distilled water. To 2 cm^3 of each extracts few drops of 2% w/v ferric chloride solution were added. Formation of a dark green color was an indication of the presence of phenolic compounds.

2.5.2 Test for Tannins

To 2 cm^3 of each extracts 1 cm^3 of distilled water and 3drops of 10% w/v ferric chloride solution were added. Formation of blue or green black color was an indication of the presences of tannins.

2.5.3 Test for Flavonoids

Lead acetate test: To 1 cm^3 of each of the extracts few drops of 10% w/v lead acetate solution were added. Formation of yellow precipitate was an indication of the presences of flavonoids.

2.5.4 Test for Terpenoids

Salkowski's test: To 1 cm^3 of each of the extracts, 3 cm^3 of chloroform was added. The resultant solution was carefully mixed with 2 cm^3cconcentrated sulphuric acid. Formation of a reddish brown colour at the interface was an indication of the presence of terpenoids.

2.5.5 Test for Glycosides

To 5 cm^3 of the extracts, 2 cm^3 of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 cm^3 of concentrated H_2SO_4. Formation of a brown ring of the interface was an indication of the presence deoxy sugar of cardenolides.

2.5.6 Test for Alkaloids

To 1 cm^3 of the extracts few drops of concentrated Hydrochloric acid and Dragendorf's reagent were added. Formation of white precipitate indicated the presence of alkaloids.

To 2 cm^3 of the extracts 2 cm^3 of dilute hydrochloric acid was added. The resultant solutions were treated with few drops of Mayer's reagent. Formation of a yellow colour precipitate was an indication of presence of alkaloids.

2.5.7 Test for Saponins

Foam test: A 2 cm^3 of extracts were shaken in the test tube for 30 seconds. Formation of foam which persisted for 10 minutes was an indication of the presence of saponins.

2.5.8 Test for Steroids

To 1 cm^3 of each of the extracts 2 cm^3 of chloroform and a few drop of concentrated sulphuric acid were added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids.

2.6 Antioxidant activity

Antioxidant activity was determined using DPPH radical scavenging assay:

The ability of extracts to scavenge DPPH radicals was determined according to a published method (Hsu *et al.*, 2007) with some modifications. A 1.5 ml of 0.1 mM DPPH solution was mixed with 1.5 ml of various concentrations (20 to 100 µg/ml) of the stem bark extracts. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as blank. The experiment was replicated in three independent assays.

Ascorbic acid was used as positive controls. The absorbance of the final reaction mixture was expressed as mean ± standard deviation of the mean of triplicate. Increased in absorbance of the reaction mixture indicates the increase in the antioxidant potential of the reaction mixture. The antioxidant activity of the stem bark extracts was expressed as IC_{50} and compared with standard.

2.6.1 Assessessent of% Inhibition and IC_{50}

Radical scavenging activity of extract and standard were expressed in terms of% inhibition. It is calculated by using the formula:

$$\% \, I = \left[\frac{A\ control - A\ sample}{A\ control}\right] \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of the sample of ethanol, petroleum ether, chloroform, ethyl acetate and aqueous extracts.

The IC_{50} value is defined as the concentration (in µg/ml) of extracts that produced 50% antioxidant effect.

$$IC50 = \left[\frac{Concentration\ of\ extracts}{\%\ Inhibition}\right] \times 50$$

2.6.2 Statistical analysis

All data were expressed as mean ± standard deviation of 3(n) measurements. Statistical analysis was performed by One Way ANOVA using manual method and confirmed by SPSS software version 2.0 and $P \leq 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1 Results

3.1.1 Extraction

The stem bark of *Diospyros mepilformis* was extracted using various solvents and the nature of crude extracts recovered is presented in Table 1 below:

Table 1: Nature and Recovery of the Crude Extracts of *Diospyros mespiliformis* stem bark.

Extracts	Texture	Colour	Wt of Sample (g)	Wt of Extracts (g)	% Recovery
Methanol	Oily, Sticky	Dark	100.00	12.50	12.50
	Paste	Brown			
Petroleum	Oily& Stick	Reddish	10.50	1.30	12.38
Ether	Liquid	Brown			
Ethylacetate	Solid.	Brown	7.50	2.40	32.00

Key: Wt of Sample is the sample weight (g), Wt of Extracts is the extracts weight

3.1.2 Phytochemical screening

Phytochemical screening was carried out on the crude extracts of *the stem bark of Diospyros mespiliformis* for qualitative determination of various secondary metabolites and the results obtained were tabulated in table 2 below:

Table 2: Phytochemical screening of the solvent extracts of *Diospyros mespiliformis* stem bark.

Secondary metabolite	ME	PE	EAE
Alkaloids	+	-	+
Phenolics	+	-	+
Tannins	+	-	+
Flavonoids	+	-	+
Saponin	+	+	+
Terpenes	-	+	-
Glycosides	+	+	+
Steroids	-	+	-

KEY: + = Presence, --- = Absence ME = Methanol extract, PE = Petroleum ether extract, EAE = Ethyl acetate extract

3.1.3 Antioxidant activity

The antioxidant activity of the crude extracts was determined by DPPH Radical Scavenging Assay and the results were presented in table 3 below:

Table 3: *In-Vitro* free radical scavenging activity of crude extracts of *Diospyros mespiliformis* stem bark by DPPH radical scavenging assay

Extracts	% I	IC_{50} (20 µg/ml)
Methanol	30.50 ± 1.23^{c}	32.8 ± 1.29^{b}
Pet. Ether	22.69 ± 0.96^{d}	43.80 ± 1.83^{a}
Ethyl acetate	32.62 ± 1.23^{b}	30.69 ± 1.28^{c}
Ascorbic acid	39.01 ± 1.23^{a}	25.65 ± 0.80^{d}

Key:% I = Percentage Inhibition I.C_{50} = Concentration (in µg/ml) of extracts that produced 50% antioxidant effect

Note: The results are Mean ± Standard deviation (n = 3). Values on the same column with the same letters are significantly the same, while values on the same column with different letters are significantly different at $p \leq 0.05$.

3.2 Discussion of Results

Table 1 shows the nature and recovery of each solvent fraction. Exhaustive extraction of 100 g sample (*Diospyros mespiliformis* stem bark) with 80% v/v methanol recovered 12.50 g of extract (12.50% w/w recovery) which was partitioned with petroleum ether (12.38% w/w recovery) and ethyl acetate (32.00% w/w recovery).

The result of the phytochemical screening of the crude extracts (table 2) revealed the presence of active entities that elicit a major pharmacological response. The result revealed the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, terpenes and glycosides. The presence of flavonoids and tannins in the methanol and ethyl acetate extracts is likely to be responsible for the free radical scavenging effects observed. Flavonoids and tannins are phenolic compounds and plant phenolics are major group of compounds that act as primary oxidants or free radical scavengers. The presence of one secondary metabolite in one solvent extract and the absence in another solvent extract might be due to difference in solvent polarity which agrees with the rule of thumb 'like dissolves like'.

The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517 nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution decolorized as the colour changes from deep violet

to light yellow. The degree of reduction in absorption measurement is an indicative of free radical scavenging (antioxidant) activities of the extracts. The scavenging activity of ascorbic acid, methanol, petroleum ether and ethyl acetate extracts of *Diospyros mespiliformis* stem bark were summarized in table 3.

Antioxidant activity was expressed based on IC_{50} values and the result range from 30.69 ± 1.28 µg/cm^3 (IP = 32.62 ± 1.23) to 43.80 ± 1.83 µg/cm^3 (IP = 22.61 ± 0.96). Ethyl acetate extract has the lowest value of IC_{50} (30.69 ± 1.28 at 20 µg/cm^3) while petroleum ether has the highest value of IC_{50} (43.80 ± 1.83 at 20 µg/cm^3). The lower the value of IC_{50} (higher IP), the better is the antioxidant activity. On the other hand, the higher the value of IC_{50} (lower IP) the lesser the antioxidant activity. Hence among the solvent extracts, ethyl acetate extract has exhibited the highest antioxidant activity (lower value of IC_{50}) followed by methanol extract and petroleum ether extract has the lowest. Although, ethylacetate has the lowest polarity index, it has higher molecular weight compared to methanol and petroleum ether. It has been noted (Kassim *et al.* 2011) [10] that the higher the molecular weight of the solvent, the lower the polarity which allows other substances of about the same molecular weight to be easily extracted (Rajan *et al.* 2011). However the activity of the extracts was found to be less when compared to the standard (ascorbic acid). IC_{50} value for standard was found to be 25.65 ± 0.80 µg/cm^3 at 20 µg/cm^3.

Statistical analysis (by ONE-WAY ANOVA) showed that there is a significant difference between the mean of various solvents extracts and the ascorbic acid (positive control). Least significant difference (LSD) test also showed that both the samples were significantly different at $P \leq 0.05$

4. Conclusion

The result of this study revealed that *Diospyros mespiliformis* stem bark was very rich in phytochemicals that are of high medicinal importance. It was also revealed that the stem bark of the plant contained natural antioxidants, which can be used as supplements to aid the therapy of free radical mediated diseases such as cancer, diabetes, inflammation, etc. It has been established that antioxidant potential of medicinal plants may be related to the concentration of their phenolic compounds which include phenolic acids, flavonoids, anthocyanins and tannin (Muanda *et al.,* 2009) [14], and since these compounds have been found to be present in the stem bark of this plant, it is not surprising that the plant possesses high antioxidant properties. Phenolic compounds and flavanoids can readily donate electrons to the free radical therefore can be used as a source of natural antioxidants since the synthetic antioxidants have been linked to different adverse effects.

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