

Phytochemicals Content and *In vitro* Antioxidant Activity of Methanol Stem bark Extract and Fractions of *Balanites aegyptiaca* Del. (Desert date) Locally used in Stroke Treatment in Kebbi

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Abstract

Balanites aegyptiaca (Desert date) has long been utilized in North-western Nigeria, particularly in Kebbi State, for the treatment of ailments, including jaundice, malaria, and heart-related conditions. However, there is currently no scientific documentation regarding its phytochemicals and *in vitro* antioxidant activities. The objective of this study was to identify the phytochemicals and assess the *in vitro* antioxidant activity of crude methanol extracts and several fractions (ethyl acetate, n-butanol, n-hexane, and chloroform) derived from *B. aegyptiaca*. Crude extraction, fractionation, as well as qualitative, quantitative, and *in vitro* tests were conducted using established protocols. The qualitative analysis of the crude methanol extract detected saponins, terpenoids, alkaloids, phenols, tannins, steroids, and flavonoids. However, cardiac glycoside and anthraquinone were not found. Some of the examined phytochemicals were present in one or the other fraction and those missing in one fraction were present in the other fraction thus validated the rationale for utilizing various solvents. The alkaloids contents of the methanol extracts (1.58 ± 0.02 mg/g) and n-hexane fraction (1.72 ± 0.04 mg/g) were significantly higher than that of the other fractions. Total tannins content in the n-butanol fraction (0.66 ± 0.03 mg/ml) was higher than all the fractions. More so, the n-hexane fraction had the highest content of flavonoids (1.12 ± 0.05 mg/g) and phenols (2.1 ± 0.05 mg/ml). The extracts and all fractions demonstrated a dose related increase in 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity as well as the Ferric Reducing Antioxidant Capacity (FRAC). This suggests that bioactive substances from *B. aegyptiaca* have significant potentials in controlling oxidative stress related disorders. *In vivo* antioxidant activity of the plant and its toxicity need to be explored.

Keywords: Antioxidants, *Balanites aegyptiaca*, DPPH, FRAC, Phytochemicals

1.0 Introduction

The world is blessed with self-borne medicinal plants. Medicinal plants are currently more in demand than ever before. It is so because with time, scientists and laymen are realizing their values and the huge properties they possess that will prove to be boon for the societies in the present and future generations. More precisely in the genres that favour the well-being of mankind, particularly in focus on medication and pharmacology [1].

Phytochemicals found in plants have a wide range of bioactive properties, including antioxidant, anti-inflammatory, and anti-cancer properties. Currently, about 25 percent of the active ingredient used in prescription drugs has been identified from plants [2].

The pharmacological property of any medicinal plant depends on the phytochemicals of such medicinal plant[3]. These phytochemicals possess arrays of properties, the most common being their antioxidant activity. This property is particularly important for phenolic and flavonoid compounds

because of their capacity to scavenge free radicals emanating from different reactive oxygen and nitrogen species (ROS/RNS)[4]. In most living cells, these reactive species (ROS/RNS) are not only produced by stress or respiration, but have also been known to be produced by exposure to radiation, by bacterial and viral toxins, by smoking, and by alcohol. If these reactive species aren't trapped or eliminated by these cellular constituents, they can lead to serious diseases [5].

Antioxidant properties are the basis of the downstream properties of medicinal plants such as anti-jaundice, anticancer, and antidiabetic among others. Natural antioxidants from plant source can protect the body from harmful free radicals and slow down the progression of many chronic diseases. Due to their effect on the human immune system, natural antioxidants agents are necessary when compared to artificial antioxidants which are harmful to humans [1].

Balanites aegyptiaca Del., also known as 'Desert date' in English, is a member of the family Zygophyllaceae, and one of the most common but neglected wild plant species of the dry land areas of Africa, parts of the Middle East and South Asia [6]. *B. aegyptiaca* Del. is used locally in Kebbi State for the treatment of various diseases like jaundice and stroke. However, conducting phytochemical investigations and *in vitro* antioxidant studies can provide a foundation for utilizing this esteemed plant. Thus, the objective of this research was to identify the phytochemicals and determine the *in vitro* antioxidant activity of crude methanol extract and fractions obtained from *Balanites aegyptiaca*.

2.0 Material and Method

2.1 Sample collection

A basic questionnaire was utilized in questioning traditional medicine practitioners about herbs used for the management/treatment of stroke. The stem bark of the plant was acquired from traditional medicine practitioners. The plant samples were botanically identified and authenticated at the herbarium unit of the Biological Science Department, Federal University Birnin Kebbi with a Voucher number of FUBK/HERB/0104.

2.2. Preparation of Methanol Extract

The stem bark was collected and air dried at room temperature and in a well-aerated atmosphere and prevented from direct sunshine to avoid denaturation of important phytoconstituents. The dried stem barks were crushed. Exactly 300g of the ground sample was soaked in a plastic rubber with 3L of methanol for 72 hours. The methanol extract was sieved out using a muslin cloth and concentrated to dryness to remove the solvents at decreased pressure using a rotary evaporator. The dried extract was kept in the refrigerator until further use.

2.3. Crude methanol extract Fractionation

The crude extracts were fractionated using liquid-liquid extraction methods [7]. This was carried out by dissolving a measure of 20g of the extracts into 400ml of distilled water and successively partitioned into fractions of n-hexane, ethyl acetate, n-butanol, chloroform in order of polarity using a separation funnel equipment. The collected fractions were concentrated in a rotary evaporator and the dried fractions were kept in the refrigerator until further use.

2.4. Phytochemical screening

2.4.1. Qualitative Phytochemical Screening

Qualitative phytochemical screening for presence of various phytochemicals was done using the procedures reported by Shemishere et al. [3]

2.4.2 Quantitative Phytochemical Screening

2.4.2.1 Determination of Alkaloids

Alkaloids were determined using the procedures reported by Garg and Grag [8]. One gram (1g) of the crude extract and its fractions respectively, were weighed into a 250 ml beaker, and 200 ml of 10% acetic acid in ethanol was added, covered, and left to stand for 4 hours. The extract was filtered and concentrated to one-quarter of its original volume in a water bath set to 100°C. Dropwise additions of concentrated ammonium hydroxide (NH₄OH) were made to the extract until precipitation was complete. The solution was allowed to settle before collecting the precipitate, which was then washed with dilute ammonium hydroxide (NH₄OH) and filtered. The residue is the alkaloid, which was dried in an oven,

weighed, and the total alkaloids were calculated using equation 1

$$\text{Alkaloids (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \text{-----} (1)$$

2.4.2.2. Determination of Flavonoids

This was done as reported by Shemishere et al. [3] with modification. At room temperature, one gram (1g) of plant material was extracted with 80% aqueous methanol (100ml). The mixture was filtered using a Whatman No1 filter paper and placed in a pre-weighed empty 250ml beaker. The filtrate was transferred to a water bath, evaporated to dryness, and weighed. The total flavonoids were estimated using equation 2.

$$\text{Flavonoids (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \text{-----} (2)$$

2.4.2.3. Determination of Tannins

This was carried out in the manner reported Shemishere et al. [3] with some adjustments. Half a gram (0.5g) of the sample was dissolved in 50ml of distilled water and shaken with an automated shaker for 1 hour. It was filtered into a 50ml volumetric flask and filled to the mark. Five millilitres of the filtrate was transferred to duplicate test tubes, mixed with 2ml of 0.1M FeCl₃ in 0.1N HCl and 0.008M potassium ferrocyanide, and left to stand for 10 minutes. The absorbance was measured using a spectrophotometer at 700nm in comparison to the blank. Tannic acid was employed as the standard at a concentration of 100mg/ml (0.1g/ml). The concentration of the sample was determined using equation 3

$$\text{Concentration of test} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard} \text{----} (3)$$

2.4.2.4. Determination of Phenols

This was determined spectrophotometrically using the Folin-Ciocalteu method as reported by Shemishere et al. [7] with minor modifications. One hundred milligrams (0.1g) of the extract was weighed, diluted in 100ml of triple distilled water (TDW), and let to dissolve fully. One milliliter of the resultant solution was put into a test tube, followed by 0.5ml of 2N Folin-Ciocalteu reagent, 1.5ml of 20% sodium trioxocarbonate IV (Na₂CO₃) solution, and triple distilled water (TDW) to fill the tube to 8ml. The mixture was vigorously shaken and allowed to stand for two hours before the absorbance was measured at

765nm. The standard was 100mg/ml of gallic acid, and the concentration of phenols was estimated using equation 4.

Concentration of test (g/ml) =

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard} \text{-----} (4)$$

2.4.2.5. Determination of Saponins

Saponins was determined as reported by Nandhini and Ilango [9]. A conical flask was filled with two grams (2g) of pulverized material and 10 millilitres (20%) of aqueous ethanol. The sample was heated in a water bath for 0.4 hours with constant stirring at approximately 550 degrees Celsius. The resultant mixture was placed to a 250 mL separating funnel, and 2 mL of diethyl ether was added and rapidly agitated. The ether layer was discarded, whereas the aqueous layer was recovered. Then, 6 mL of n-butanol was added, followed by 1 mL of 5% aqueous sodium chloride (NaCl). The residual solution was heated on a water bath. After evaporation, the sample was oven-dried to a consistent weight, and the total saponins were determined using equation 5.

$$\text{Saponins (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \text{-----} (5)$$

2.5. In vitro antioxidant analyses

The antioxidant activities of *Balanites aegyptiaca* methanol extracts and fractions were quantified using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, and Ferrous reducing antioxidant capacity (FRAC).

2.5.1 Ferrous reducing antioxidant capacity assay

The ferric reducing antioxidant capacity (FRAC) of the samples was tested by the method reported by Shemishere et al.[3]. The Fe²⁺ was monitored by monitoring the production of Perl's Prussian blue at 700 nm. Exactly 0.25 mL samples/standard solution at varied concentration (12.5–150 µg/mL), 0.625 mL of potassium buffer (0.2 M) and 0.625 mL of 1 % potassium ferricyanide (K₃Fe CN) solution were put into the test tubes. The reaction mixtures were incubated for 20 min at 50 °C to complete the reaction. Then 0.625 mL of 10 % trichloro acetic acid (TCA) solution was put into the test tubes. The complete combination was centrifuged at 3000 rpm for 10 min, after which 1.8 mL supernatant was removed

from the test tubes and mixed with 1.8 mL of distilled water and 0.36 mL of 0.1 % ferric chloride (FeCl₃) solution. The absorbance of the solution was measured at 700 nm using a spectrophotometer against blank (equation 6).

% inhibition

$$\frac{(Absorbance\ of\ sample - Absorbance\ of\ control)}{(Absorbance\ of\ sample)} \times 100 \text{---(6)}$$

2.5.2 Estimation of Diphenyl-2-Picryl-Hydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging capacity of the extracts against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by a slightly modified method reported by Madhuet al.[10]. The assay is based on the ability of the antioxidant compounds to reduce DPPH by donation of hydrogen resulting in color change from deep violet to golden yellow. The change in color from deep violet to pale yellow was measured with spectrophotometer at 517nm. In this, 2 mL of varied concentrations (0.2 - 1.0 mg/mL) of the standard, extracts, or fractions were added to 0.5 mL of a 0.3 mM DPPH solution in methanol. After 15 minutes of stirring and incubation at room temperature in the dark, the reaction tubes' absorbance was measured at 517 nm. Every test

was performed in triplicate. Ascorbic acid was generated in test samples' concentrations and used as a standard control. In order to simulate the test samples, a blank containing 0.5 mL of 0.3 mM DPPH and 2 mL methanol was created. Equation 7 was used to compute the radical scavenging activity.

%DPPH radical inhibition

$$= \left[\frac{(Absb.\ of\ DPPH+methanol) - (Absb.\ of\ DPPH+Sample/std)}{(Absorb.\ of\ DPPH+Methanolsample)} \right] \times 100 \text{---(7)}$$

3.0 Results

3.1 Qualitative phytochemicals screening results

Qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids and phenols in all extracts and all the fractions. However, anthraquinones were only detected in the ethyl acetate, n-butanol and the n-hexane fractions, cardiac glycoside in only n-hexane fraction, tannins not detected in chloroform fraction, while terpenoids were not detected in n-hexane fraction.

Table 3.1: Qualitative phytochemical constituents of methanol extract, ethyl acetate, n-hexane, n-butanol and Chloroform fractions of *Balanitesa egyptiaca*

Phytochemicals	Methanol	Ethyl acetate	n-butanol	n-hexane	Chloroform
Flavonoids	+	+	+	+	+
Anthraquinones	-	+	+	+	-
Alkaloids	+	+	+	+	+
Saponin	+	+	+	+	+
Cardiac glycoside	-	-	-	+	-
Tannins	+	+	+	+	-
Terpenoids	+	+	+	-	+
Steroids	+	++	+	+	+
Phenols	++	+	+	+	+

Keys: (+)= present

(-)= Absent

3.2 Quantitative content of selected phytochemicals

Quantitative analysis of selected phytochemicals is shown in table 3.2 below. Alkaloids, saponins,

flavonoids and phenols were high in the methanol and n-hexane fractions compared to the other fractions. Moreso, high contents of tannins were recorded in the n-butanol and n-hexane fractions.

Table 3.2. Quantitative constituents of some selected phytochemicals in methanol extract, ethyl acetate, n-butanol, n-hexane and Chloroform fractions of *Balanites aegyptiaca* in (mg/g and mg/ml)

Phytochemical	Methanol Extract	Ethylacetate	n-butanol	n-hexane	Chloroform
Alkaloids (mg/g)	1.58±0.02	1.05±0.04	0.79±0.01	1.72±0.04	0.49±0.02*
Tannins (mg/ml)	0.34±0.05	0.09±0.03*	0.66±0.04*	0.64±0.03*	0.00±0.00
Saponins (mg/g)	0.71±0.01	0.05±0.01*	0.67±0.05	0.74±0.02	0.07±0.001*
Flavonoids (mg/g)	1.10±0.01	0.09±0.01*	0.41±0.04*	1.12±0.05	0.07±0.001*
Phenol (mg/ml)	0.45±0.03	0.09±0.01*	0.31±0.03	2.1±0.05*	0.09±0.001*

Values are mean ± standard deviation of duplicate determination, values in the same row having * superscript are statistically significant when compared to the methanol extract at ($p < 0.05$)

3.3 In vitro Antioxidant Activity

3.3.1 1, 1-diphenyl-2-picrylhydrazyl(DPPH) free radical scavenging activity

The DPPH radical scavenging activity revealed a dose dependent percentage inhibition increase of the extract and all the fractions.

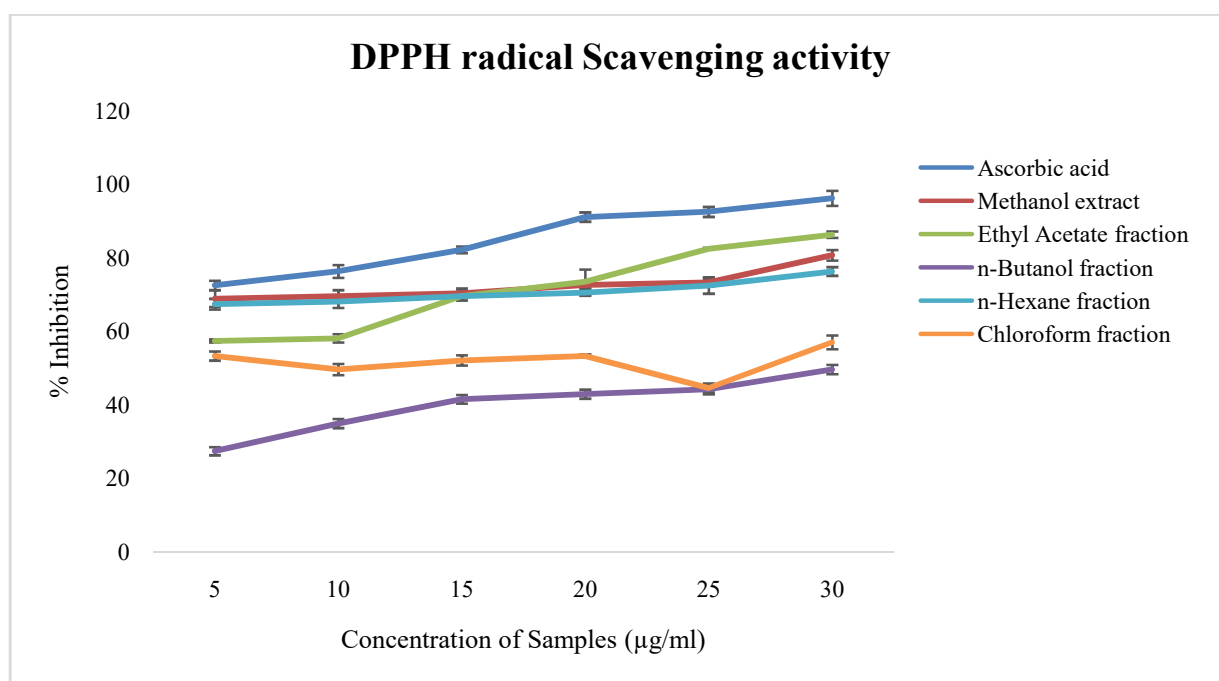


Figure 3.1: Percentage Diphenyl-2-Picryl-Hydrazyl (DPPH) Radical Scavenging Activity of ascorbic acid (standard), methanol extract, ethyl acetate, n-butanol, n-hexane and Chloroform fractions of *Balanites aegyptiaca*.

3.3.2 Ferric Reducing Antioxidant Capacity

Results from the ferric reducing antioxidant capacity (FRAC), revealed that there was no

significant difference between the reducing capacity of the standard ascorbic acid and the plant extract and fractions. This is shown in table 3.3 below.

Table 3.4: Ferrous Reducing Antioxidant capacity (FRAC) of methanol extract, ethyl acetate, n-butanol, n-hexane and Chloroform fractions of *Balanites aegyptiaca* in mg/g

Conc. in mg/g	Ascorbic Acid	Methanol extract	Ethyl Acetate	n-butanol	n-hexane	chloroform
5	0.08±0.01	0.07±0.01	0.07±0.01	0.13±0.01	0.07±0.01	0.05±0.02
10	0.09±0.01	0.07±0.01	0.08±0.01	0.11±0.08	0.08±0.01	0.06±0.02
15	0.12±0.02	0.08±0.02	0.12±0.02	0.11±0.06	0.10±0.02	0.07±0.02
20	0.14±0.02	0.09±0.01	0.14±0.01	0.03±0.06	0.12±0.02	0.08±0.01
25	0.17±0.03	0.12±0.02	0.10±0.02	0.15±0.08	0.14±0.01	0.10±0.02
30	0.15±0.02	0.15±0.02	0.11±0.03	0.09±0.03	0.1±0.003	0.08±0.03

Values are mean ± standard deviation of duplicate determination, values in the same row having * superscript are statistically significant when compared to the methanol extract at ($p < 0.05$).

4.0 Discussion

Phytochemical analysis is capable of recognizing distinct groups of chemicals in a sample based on findings such as changes in colour or precipitate development [2,7]. The preliminary qualitative phytochemicals screening of methanol bark crude extract of *Balanites aegyptiaca* and fractions revealed the presence of flavonoids, phenols, and terpenoids, and the absence of anthraquinone and cardiac glycoside in crude methanol extract.

Screening plant materials for their phytochemicals is usually the first step in research protocol aimed at the isolation and purification of natural compounds with bioactivities [11]. It allows the researcher to see at a glance the phytochemicals present in a plant material and, hence, gives an idea of the potential use of the plant material [4]. These phytochemical constituents have been shown to protect the plant from infection.

Quantitative phytochemical constituent estimation provides significant information on the concentration of the individual phytochemical in question [4]. In this study, an array of different phytochemicals was identified and quantified. These phytochemicals have been linked to therapeutic benefits and could have a combined effect on the many pharmacological claims made for *Balanites aegyptiaca*. Plants contain phenols in large quantities, and they have been shown to have a variety of biological functions, including the ability to effectively scavenge free radicals [1]. Antioxidant, anticancer, antibacterial, anti-inflammatory, and anti-clotting properties have been documented for phenols and phenolic compounds [2]. Because they can get rid of free radicals, they can prevent a lot of ailments. Among the phenols, flavonoids are the most common category. They demonstrate a wide range of pharmacological and chemical actions. The

following characteristics of flavonoids: diuretic, anti-spasmodic, hypocholesterolemic, hepatoprotective, hormone modulator, antimicrobial, cytotoxic, anti-inflammatory, immunological enhancer, and vascular [12].

Flavonoids have been reported to have strong antioxidant properties, with the ability to scavenge a wide range of free radicals, inhibit lipid peroxidation and are used for therapeutic purposes. Tannins are polyphenolic phytochemicals with reported pharmacological benefits including anti-inflammatory, anti-oxidant, anti-tumor, anti-infective, blood clotting, hypolipidemic, immunomodulatory and antimicrobial [12]. Phenolics are the most common antioxidants present in human diet. The phenol group constituent of all polyphenolics imparts the antioxidant property on plants containing them. The results of this study are in agreement with that from Murthy et al. [13] who reported that the seeds and leaves of the plant are rich in the identified phytochemicals.

The result of *in vitro* antioxidant studies showed a dose dependent increase in the DPPH free radical scavenging activity in standard ascorbic acid and in all the fractions and methanol extract. This is line with the findings of Usman et al. [14] and Berihu et al. [15] who reported the *in vitro* antioxidant activities of the ethanol stem bark extracts of *Balanites aegyptiaca*. The antioxidant capacity of phenols and polyphenols such as flavonoids, tannins, and others is thought to be conferred by their hydroxyl group(s) (OH), which is directly linked to an aromatic hydrocarbon (phenyl ring). As a result, they can easily donate electrons to electron-seeking free radicals, as a result, their effects on living cells are reduced [16]. The results revealed that the methanol extract and fractions of *Balanites aegyptiaca* have

a moderate free radical scavenging activity when compared with standard ascorbic acid.

The total antioxidant capacity of biological fluids may be determined with the easy, quick, and accurate FRAC test [3,17]. According to Katalinic *et al.* [18], the FRAP test is linearly correlated with the molar concentration of the antioxidant contained in the biological sample and is repeatable. A compound's ability to reduce can be a useful measure of its possible antioxidant activity. This is because the Fe^{3+} ferricyanide complex is reduced to the ferrous form in the presence of reductants, such as antioxidant compounds [19]. Interestingly, the FRAC assay results demonstrated that the extracts and fractions had reducing capacities that were comparable the standard ascorbic acid. The observed antioxidant potentials of the extracts and fractions of *B. aegyptiaca* may be attributed to the array of phytochemicals that were quantified in the samples.

5.0 Conclusion

This study's findings indicate that the screened phytochemicals are dispersed unevenly across the n-hexane, ethyl acetate, n-butanol and chloroform fractions of the *B. aegyptiaca* stem bark methanol extract. The methanol extract and n-hexane fraction contained more alkaloids, phenols, saponins, tannins and flavonoids than other fractions. The antioxidant capacity and DPPH free radical was scavenged by all fractions and extract in a concentration-dependent manner. However, this ability may be linked to the various phytochemicals that were identified in the samples. In order to utilize these particular phytochemicals for the treatment of diseases associated to oxidative stress, more research into them which will focus on *in vivo* antioxidant capacity is advised.

6.0 Conflict of interest

The authors declared no conflict of interest.

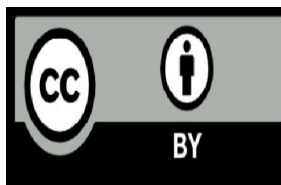
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