Nigerian Research Journal of Chemical Sciences (ISSN: 2682-6054)
Volume 13, Issue 1, 2025

Total Phenolic, Flavonoid Contents and Antioxidant Activity of Stem Bark Extracts of *Ziziphus mauritiana*

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ABSTRACT

Phenolics and flavonoids in plants possess essential biological activities making it necessary to analyze them. Antioxidants have the ability to scavenge radicals. In this study, extracts from the stem bark of *Ziziphus mauritiana* were screened. The powdered plant material was extracted using ethanol by Soxhlet extraction method and fractionated into n-hexane, chloroform, ethyl acetate and aqueous methanol fractions. The total flavonoids were determined spectrophotometrically using an aluminium chloride colourimetric assay. The antioxidant activity was determined by measuring the radical scavenging effect of the extract/fractions and ascorbic acid on the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical. The Folin Ciocalteu assay was used to measure the phenolic contents. The results showed that the extract/fractions contained phenolics. The highest total flavonoid content was revealed in chloroform fraction (57.24 mg/g QEqv) while ethanol fraction had the lowest 22.69 (mg/g QEqv). The antioxidant activity, expressed as IC_{50} values, varies from 0.002 µg/ml in ethanol extract to 99.021 µg/ml in n-hexane fraction. The results showed that the ethanolic and methanolic fractions have higher phenolic content with 363.67 and 553.93 µg/mg gallic acid equivalents respectively. The results showed that the plant has antioxidants that may be responsible for its ethno-medicinal uses. Further research such as spectroscopic analysis is recommended to elucidate the structure of bioactive compounds present in the plant.

Key words: Phenolics, flavonoids, antioxidants, DPPH, *Ziziphus mauritiana*, Folin-Ciocalteu, ethanol, methanol.

INTRODUCTION

Medicinal plants are crucial for the wellbeing of the population [1]. For centuries, plants have served as a valuable source of therapeutic agents, with their diverse secondary metabolites displaying a wide range of pharmacological activities [2]. The use of plants and plant-based products to meet societal health needs stems from the safety and cost effectiveness of the

utilizing plants in traditional and modern medicine [3], and high cost, adulteration and increasing toxic side effects of synthetic drugs [4]. Natural antioxidants, such as carotenoids, ascorbic acid, tocopherols, and flavonoids, can protect the human body from free radicals. They also help slow and the progression of many chronic diseases and prevent lipid oxidative rancidity in foods [5].

The antioxidative properties of flavonoids are attributed to several mechanisms, including scavenging free radicals, chelatiing metal ions, like iron and copper, and inhibiting enzymes that generate free-radicals [6]. Hence, flavonoids may protect biosystems against free-radical attack, which may be involved in various cancers and coronary heart disease.

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease [7].

Ziziphus mauritiana, a member of the family *Rhamnaceae,* is a fruit tree that grows in tropical and sub–tropical regions of the world. Different parts of this plant have been used in traditional medicine for the treatment of various ailments such as asthma, allergies, depression and ulcers [8]. The leaves are considered diaphoretic and are prescribed for typhoid in children. They are also used as poultices. A decoction of the bark of *Z. mauritiana* is used for the treatment of diarrhoea and dysentery. The bark is also used as an astringent in gingivitis. *Z. mauritiana* root is used to cure headache. Decoction of roots is used in fever, and as a powder applied to old wounds and ulcers [9]. Some studies have also investigated the phenolics composition of the fruit and illustrated the scientific basis for the uses of different parts of this plant for the treatment of diabetes, ulcers and inflammation [10]. *Z. mauritiana* also possesses anti–diabetic, anti – Inflammatory, anti–plasmodial, and anti–microbial, as well as hemolytic, sedative, anxiolytic, diuretic, analgesic and antioxidant properties [11].

Research has identified a diverse range of compounds within different parts of the plants, including fruits, seeds, bark and roots. These compounds include betulinic aldehyde, betulinic acid, ceanothic acid, frangufoline, spinosin, beta –sitosterol, daucosterol, daucosterol – 6'–octadecanoate, docosanoic acid , stearic acid and palmitoleic acid [12]. Studies have shown that these compounds are linked to anti-inflammatory and anticancer properties [13]. Studies have also reported the presence of sugars like sucrose, D-ribofuranose and volatile compounds like furfural derivatives in *Z. mauritiana* [14].

The rise of antibiotic resistant bacteria as a result of the overuse and misuse of antibiotics poses a threat to human health. Despite their traditional medicinal uses a comprehensive understanding of the specific bioactive components present in plants is worth pursuing. In this study, DPPH radical scavenging activity, the phenolic and flavonoid contents of extracts of the stem bark of *Z. mauritiana* were analyzed.

The study aims to investigate the antioxidant activities of various fractions obtained from the stem bark of *Ziziphus mauritiana* to confirm their ethno-medicinal uses and explore their potential as ingredients for the development of natural drugs.

The specific objectives of this study are:

1. To collect, prepare and extract the air-dried parts of the plant with ethanol using Soxhlet extraction.
2. To partition the plant extract using solvents of different polarities (ethanol, aqueous methanol, ethyl acetate, chloroform, and n-hexane)
3. To subject the fractions obtained to total flavonoid content, total phenolic content and DPPH radical scavenging antioxidant assays.

MATERIALS AND METHODS

Materials

Folin-Ciocalteau's reagent, sodium carbonate (Na_2CO_3), L-ascorbic acid (vitamin C), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid (GA). Soxhlet extractor, was used for extraction of samples and Rotary evaporator (Buchi Rotavapor II, Buchi, Switzerland). All other chemicals used were of analytical grade. All glass wares were obtained from local glass ware shops.

Plant Sample Collection

Ziziphus mauritiana stem barks were collected at Kachako, Kano State, Nigeria. A voucher specimen with the accession number BUKHAN 0223 was deposited at the herbarium of the Department of Biological Sciences, Bayero University, Kano, Nigeria. The samples were washed with water to remove soil debris, chopped and dried for several days in the shade. After drying, they were ground into a powder using a mortar and pestle [15], and then sieved.

Extraction

The powdered sample (100 g) was packed into a muslin cloth tied at one end to form a bag, which was then placed in a Soxhlet extractor containing 400 ml of ethanol. The sample was

extracted for 8 hours at 40 ^{0}C. The extracts were filtered using Whatman filter paper (No. 1). The resulting extracts were then concentrated under vacuum using **a** rotary evaporator (Buchi Rotavapor II, Buchi, Switzerland) at 40 ^{o}C and reduced pressure [16] and allowed to dry at open air to give the crude extract.

Fractionation

Some portions of the crude plant extract were preserved while the rest was separated using solvents of varying polarities (n-hexane, chloroform, ethyl acetate and aqueous methanol) in increasing order of polarity. The resulting fractions were concentrated, dried and labeled accordingly.

Antioxidant Assay

Sample fractions were screened spectrophotometrically for antioxidant activities via DPPH radical scavenging assay, total phenolic content, and total Flavonoids content.

DPPH Radical Scavenging Assay.

The method described by Aktumsek et al [17] was employed to measure the free radical scavenging activity of the plant was but with some modifications. Exactly 100 µL of the sample fractions (1000, 500, 125, 62.5, 31.25, 15.625, and 7.8 µg/ml) were prepared on a 96 well micro plate and absorbance (Abs_{blank}) was measured at 517 nm. 2 00µl DPPH (100 mM) solution was added to the sample and the resulting mixture was incubated for 30 min at 25^{0}C. The absorbance (Abs_{sample}) of the resulting solution was measured at 517 nm. A solution of 200 µl DDPH and 100 µl methanol was used as control, and absorbance ($Abs_{control}$) was also measured at 517 nm. The colour change is measured quantitatively by the principle of colour change of the DPPH solution from purple to yellow as the radical is scavenged by the antioxidant. This is by spectrophotometer absorbance at 517 nm using Thermo-Scientific Multiskan Go spectrophotometer (IC 262810489).

Absorbance of DPPH (200 µL) and methanol (100 µL) was used as the control ($Abs_{control}$). Ascorbic acid and Butylated hydroxy toluene (BHT) were used as standards. Antioxidant activity was expressed as percentage inhibition using $I\ \% = 100 - \left(\left(\frac{AS-AB}{AC}\right) * 100\right)$

Where AS is absorbance of sample with DPPH; AB is absorbance of sample without DPPH
AC is absorbance of control (DPPH)

IC_{50} was computed using SPSS software.

The radical scavenging activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%

Total Phenolic Content

The total phenolic contents of the sample fractions were determined by using Folin-Ciocalteu procedure in which gallic acid was used as a reference standard for plotting calibration curve. Approximately 0.1 ml of the plant extract (100 µg/ml) was mixed with 1 ml of diluted Folin-Ciocalteu reagent (diluted 1:10 with distilled water). 1 ml saturated sodium carbonate solution was added to the mixture. The reaction mixture was incubated at room temperature for 30 min. Exactly 300 µl was pippeted into 96 microplate and the absorbance was measured at 765 nm using micro plate reader. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The total phenolic contents were expressed as mg/g gallic acid equivalent.

Total Flavonoid Content

Aluminium chloride assay was used to determine the total flavonoid content of the extracts. Approximately 0.5 ml of the extracts were taken in different test tubes. Then 2 ml of distilled water was added followed by the addition of 0.15 ml of sodium nitrite (5% $NaNO_2$, w/v) and allowed to stand for 6 min. 0.15 ml of aluminium chloride (10% $AlCl_3$) was added and incubated for 6 min, followed by the addition of 2 ml of sodium hydroxide (NaOH, 4% w/v) and the volume was made up to the 5 ml with distilled water. After 15 min of incubation the mixture turned to pink whose absorbance was measured at 510 nm using a spectrophotometer. A standard curve of quacertin was prepared and the flavonoid content is expressed mg/g quacertin equivalent.

RESULTS AND DISCUSSION

The DPPH test is a simple method for screening antioxidant molecules because the intensity can be analysed by simple spectrophotometric assays. The DPPH radical is scavenged by antioxidants through the pairing of odd electron of the nitrogen to form a non-radical product (Figure 1). The DPPH assay provides information on the reactivity of the tested extracts with

stable free radicals which causes biological damage through oxidative stress and such process lead to many disorders like neurodegenerative diseases and cancer [18] .

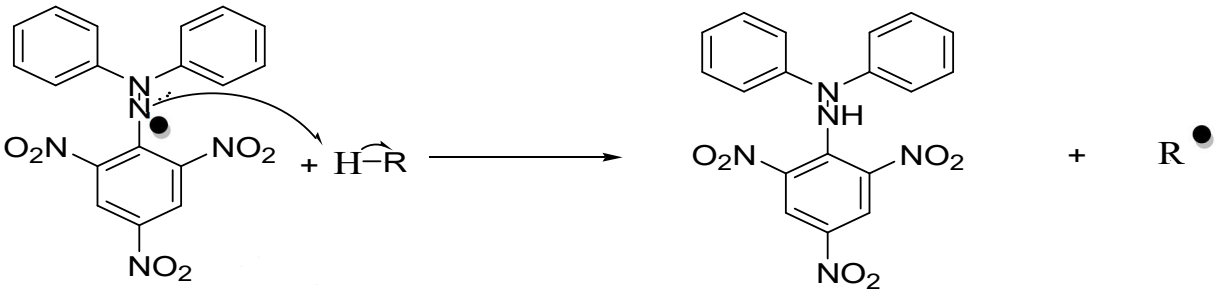

Figure 1: DPPH Reaction

DPPH assay is based on colour change of the DPPH solution from purple to yellow as the radical is scavenged by the antioxidant. The colour change is then measured quantitatively by spectrophotometer absorbance at 517 nm [19]. The percentage antioxidant activity of the plants extracts were plotted as a function of concentration in comparison with ascorbic acid and butylated hydroxyl toluene (BHT). The scavenging effect of the plant extracts from *Z. mauritiana* (Figure 2) , ascorbic acid (AA) and butylhydroxytoluene (BHT) on DPPH radicals were expressed as percentage inhibition. The amount of antioxidant needed to decrease the initial concentration of DPPH by 50% (IC50), statistically significant at $P<0.05$ samples compared with the AA and BHT are shown in Table 1. However, the IC50 changes according to the final concentration of the DPPH. The scavenging activity was measured using serial dilution of the concentrations 1000–15.63 µg/mL, the ascorbic acid (IC50=1.03 µg/mL) and butylhydroxytoluene (IC50 = 0.89 µg/mL) were used as positive control. The results showed that ethanol extract of bark of *Ziziphus mauritiana* gave higher activity of (IC50 = 0.02 µg/mL) in comparison with the ascorbic acid and butylhydroxytoluene, while the other extracts were found to be lower than the positive control (Table 1). *Z.mauritiana stem* bark extracts contains a comparable antioxidant to the positive control, and suggests the presence of secondary metabolites with readily abstractable hydrogen in the extracts.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants. It has been proposed that the antioxidant potentials of phenolic

compounds can be mediated by the following mechanisms: scavenging free radical species; suppressing free radicals formation by inhibiting some enzymes or chelating trace metals involved in free radical production; and the regulating of antioxidant defense [20]. The total phenolic content of extracts from *Z. mauritiana* were investigated based on Folin-Ciocalteu's method. A standard curve of Gallic acid was prepared (Figure 3) and the phenolic content is expressed as mg gallic acid equivalent/g of extract (mg prepared GAE/g; $y = 0.003x$; $r^2 = 0.988$) as displayed Table 1.

The Folin-Ciocalteu assay has been used to measure phenolic contents, with the basic mechanism of electron transfer and reducing ability. The amounts of total phenolic in the extracts of *Z. mauritiana* stem were found in the order of ethanol, aqueous methanol, ethyl acetate, chloroform and n-hexane , and methanol extract from the stem exhibited higher phenolic content 553.93 ± 10.67 mg/g GAE than all other extracts of the stem (Table 1).

Flavonoids are a large group of plant secondary metabolites that have a variety of health benefits. They are antioxidants that can help protect cells from damage caused by free radicals. They may also help to reduce inflammation, improve blood circulation, and boost the immune system. The structure of a flavanoids molecule plays a role in its antioxidant activity [21]. The beneficial effect of flavanoids compounds can be mediated by several mechanisms: Free radical scavenging, metal chelation, enzymatic regulation and singlet oxygen quenching. The specific mechanism involved in a particular case may depend on the type of flavanoids and the cellular environment [22]. The total flavanoids content of extracts from *Z. mauritiana* and were investigated based on aluminium chloride assay. A standard curve of quacertin was prepared (Figure 4) and the Flavonoids content is expressed as mg quacertin equivalent/g of extract (mg QE/g; $y = 0.003x + 0.05$; $r^2 = 0.973$) as displayed in Table 1.

The amounts of total flavanoids in the extracts of stem of *Z. mauritiana* were found in the order of ethanol, aqueous methanol, ethyl acetate, chloroform and n-hexane for the stem bark extracts, and among the extracts the chloroform extracts of stem ZMS (F4) exhibited higher flavanoids contents of 66.02 mg QE/g (Table 1).

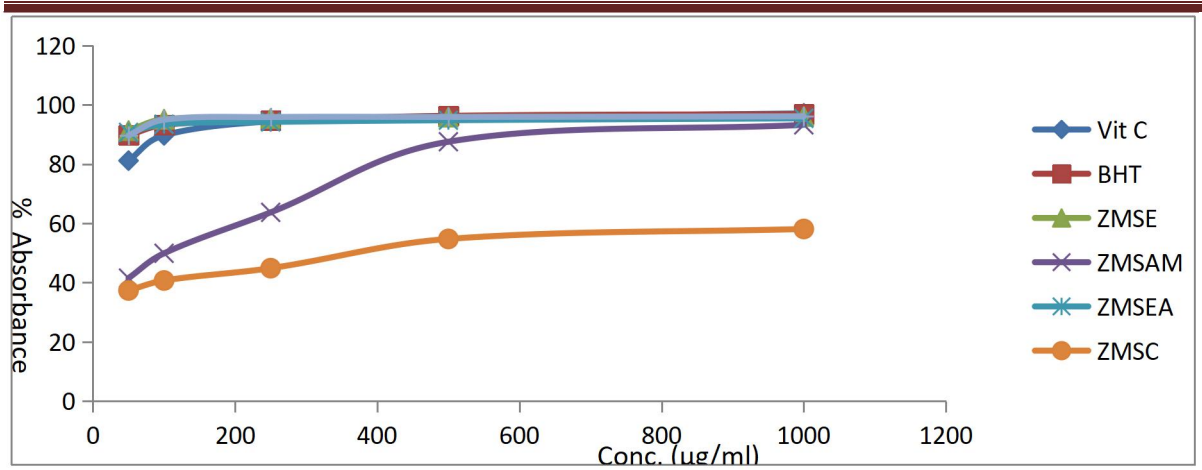

Figure 2: Percentage inhibition of DPPH radical assay of crude extracts of stem *Z.mauritiana*

KEY: ZMSE, ZMSAM, ZMSEA, ZMSC: *Ziziphus mauritiana* stem Crude Ethanol, Aq.methanol, Ethyl acetate, and Chloroform Extracts respectively.

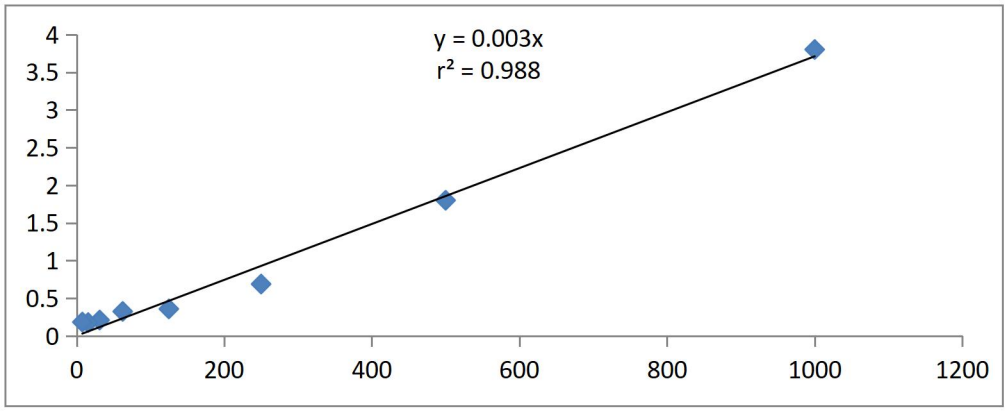

Figure 3: Gallic acid calibration curve of total phenolic contents for the extracts

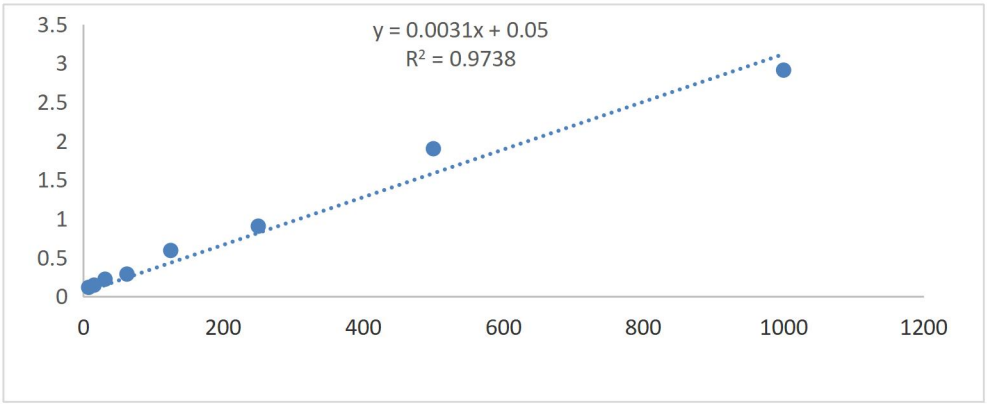

Figure 4: Quercetine calibration curve of total Flavonoid contents for the extracts

Table 1: Total Flavonoids Contents (TFC), Total Phenolic Contents (TPC), and IC_{50} Values of Extracts from the stem bark of *Ziziphus mauritiana* (ZMS)

SAMPLE FRACTION	ZMS		
	TFC (mg/g QEqv)	TPC(mg/g GAEqv)	IC_{50} (µg/ml)
F1	22.69	363.67	0.002
F2	29.92	553.93	9.411
F3	37.37	326.85	1.300
F4	57.24	93.94	33.415
F5	39.26	95.58	99.021

KEY: ZMS; *Ziziphus mauritiana* stem, F1, F2, F3, F4 and F5: Crude Ethanol, Aq.methanol, Ethyl acetate, Chloroform and n-hexane fractions respectively.

Plant samples containing high levels of gallic acid or any compound equivalent to that may be able to scavenge excessive free radicals such as superoxide anion radicals and peroxyl radicals in human body and protect cells or tissues against oxidative stress [23]. These results revealed that the solvent influences the extractability of the phenolic compounds. The phenolic extracts of plants are always a mixture of different classes of phenols, which are selectively soluble in the solvents. The use of an alcoholic solution provides satisfactory results for the extraction process [24]. Alcoholic solvents are the best solvents for extraction of phenolic compounds from stem bark of *Z. mauritiana*. This is in agreement with what Zohra [24] reported. The reduction capability of DPPH is determined by the decrease in its absorbance induced by antioxidants. Results were reported as IC_{50}, which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radical under experimental conditions. The extract that required the lowest concentration to positive DPPH test suggests that the samples were free radical scavengers. A high DPPH radical scanvenging activity is associated with a lower IC_{50} [25].

In this study, the IC_{50} result showed that alcoholic extract of the stem of *Ziziphus mauritiana* scavenged 50% DPPH radicals at low IC_{50} values. This is in agreement with what Zouhra [24] reported that alcohol solvents are the best solvents for extraction of phenolic compounds. These results established the traditional claim of the uses of the studied plants.

CONCLUSION

Based on the research conducted, it can be concluded that plant extracts have varying levels of flavonoids and phenolic contents. The IC_{50} results indicate that *Ziziphus mauritiana* stem extracts possess radical scavenging activity, suggesting the presence of secondary metabolites in the plant that support its traditional use for treatment of diseases.

RECOMMENDATION

Further evaluation of the extracts using various bioassays such as: anti-microbial, anti-HIV, anti-tumor, anti-hypertensive and anti-diabetic activities is recommended for future studies. Additional research on the plant using high-throughput screening in order to isolate and quantify compounds is also suggested.

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