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PHYTOCHEMICAL SCREENING, *In vitro* ANTIOXIDANT AND GC-MS ANALYSIS OF *Ziziphus mauritiana* LEAVES EXTRACT

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ABSTRACT

Ziziphus mauritiana (Z. mauritiana) is a plant known for its vast nutritional and pharmacological advantages. In the present investigation, leaf extracts of the plants with different solvents were analyzed by colorimetric methods for phytochemicals analysis, spectrophotometrically for the antioxidant activity by DPPH scavenging activity and gas chromatography-mass spectrometry (GC–MS) to identify the important functional groups and phytochemical constituents. The study observed the presence of some bioactive phytochemical constituents such as saponins, tannins, alkaloids, phenolics, terpenoids and flavonoids compounds. Colorimetric method suggested that, chloroform extract had the highest content of total phenolics (84.60 ± 0.92 µg GAE/mg of extract) while methanol extract contained higher content of total flavonoids (46.91±1.54 µg QE/mg of extract).Further, methanol extract exhibited higher DPPH free radical scavenging potential of 94.47% compared to ethyl acetate extract which showed the lowest activity of 70.34%.The GC–MS analysis detected the presence of 33 phytochemical compounds with diverse chemical structures from the different extracts. Major compounds identified in methanol, ethyl acetate, hexane and chloroform extracts were squalene (30.08%), salviolinic acid (12.55%), α-linolenic acid (26.42%) and palmitic acid (38.50%) respectively in addition to several other bioactive compounds.The results obtained in the present study suggest that leaves of *Z. mauritiana* can be used as a source for functional ingredients for pharmaceutical and food industries.

Keywords: DPPH scavenging activity, GC–MS, phytochemical analysis, *Ziziphus mauritiana*

INTRODUCTION

Ziziphus mauritiana, belongs to family *Rhamnaceae,* which comprises of around 100 species (Medan & Schirarend, 2004) , predominantly distributed within the tropical and sub-tropical regions of the world (Zhao et al., 2008). The family Rhamnaceae consists of deciduous or evergreen trees and climbers, shrubs and herb with thorns on the branches (Richardson et al., 2000) , and are used as a hedge to form defensive fences from animal (Orwa et al., 2009) . *Z. mauritiana* is otherwise called zizouf, Ber, Malay apple, Indian jujube, Indian plum, Jujube, desert apple, and Chinese apple (Warrier, 1993). The family Rhamnaceae are also versatile plants used as foods, folk medicines and environmental protector (Guo et al., 2017).

The plant is an evergreen spiny shrub or small tree of up to 10 m in height, branches are hanging with pointed stipules, the leaves are 2.5–4.0 cm long and 1.8–3.8 cm wide, the flowers are protandrous with five petals which are small white, yellow, or greenish-white. The fruits are edible drupe; very soft, fleshy, crispy, and acrid usually very sweet and

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sugary in taste, red, yellowish-brown, or white color, globose or oblong along which can be 2–5 cm long with apple-like aroma and pleasant smell; the skin is smooth or rough, glossy, thin but tough. Seeds are tuberculate with irregular furrowed stone, containing 1-2 elliptic brown kernels each 6 mm long (Orwa et al., 2009).

The dietary fiber and fructose contents in *Z. mauritiana* fruit slows down digestion thereby help to regulate blood sugar level (Gusakova et al., 1999) . The major sugars found in *Z. mauritiana* fruit are glucose, fructose, sucrose, rhamnose, and sorbitol. It is also abundant in vitamin C, which is one of the water soluble antioxidants (Li et al., n.d.). Furthermore, to a lesser extent, *Z. mauritiana* is enriched with other vitamins such as thiamin, riboflavin, vitamin B_6, niacin, and vitamin A. Also, it is a good source of minerals such as potassium, sodium, phosphorus, magnesium, and zinc (Li et al., n.d.; Pareek, 2017).

The leaves of *Ziziphus* species were suggested to be efficient agents for bleaching face and neck, and used in treatment of stunted hair growth (Chen et al., 2017) . The leaves are also reported to be boiled and consumed as tea (Zhao et al., 2008). In Indonesia and some part of North Africa, the leaves are cooked as a vegetable or used as nutritious ovine and caprine feed (Orwa et al., 2009) . The leaves are also used as poultices and treatment of liver problems, asthma and fever (Jain et al., n.d.) , high blood pressure and diabetes mellitus (Meena et al., n.d.). Furthermore, the leaves and roots are used for the prevention and treatment of skin diseases (Abalaka et al., 2010; Adzu et al., n.d.). The hepatoprotective activity of ethanol extract of *Z. mauritiana* leaf against CCl4 - induced liver damage in rats and the antidiarrhea activity of the methanol root extract have been reported (Dahiru et al., 2005, 2006) . The use of *Z. mauritiana* as phytomedicine for the treatment of several ailments along with the nutritional value has made it a plant of interest to researchers. To date, there is no single study that compressively determine the various phytochemicals and their biological activities as well as functional distinctions of Nigerian species of *Z. mauritiana* leaf extracts of different solvents. Therefore, this study aims to investigate and compare the phytochemicals and their biological/antioxidant activities of local species of *Z. mauritiana* leaf extracts of different solvents with the view to explore their potential uses as novel therapeutic options and/or nutraceuticals.

MATERIALS AND METHODS

Collection of Plant Materials

Fresh leaves of *Z. mauritiana* Lam. was collected from the Nigerian Defence Academy, Igabi local government area (10.6155° N, 7.3663° E) of Kaduna State, Nigeria. The plant was characterized and authenticated at the Department of Biological Sciences and voucher specimen was submitted at the herbarium. Using tap water, the leaves were gently washed and then rinsed with sterile distilled water, after which were air-dried at room temperature. The dried leaves were ground to powder using an electric blender and stored in airtight glass containers.

Preparation of Plant Extracts

The cold maceration method was utilized to extract the plant's phytochemicals. Ten grams (10 g) of the leaf powder was soaked in 100 ml of each of different solvents (methanol, hexane, chloroform, ethyl acetate and water) in separate sterile conical flask and and vigorously mixed using vortex. The mixture was then incubated in a shaker incubator (Biosan ES-20/60,Latvia) at 45^{o}C temperature with 50 rpm for 48-72 hrs. The mixtures were then filtered using Whatman filter paper grade

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followed by centrifugation (Bioevopeak CFG-5B, China) at 5000 rpm for 15 minutes. The centrifuged extract was re-filtered using 0.45 µm Millipore filter paper. The supernatant was kept for evaporation in a glass petri dish at 45°C for 24 hrs. After evaporation of solvents, the dried extracts were scraped using a spatula. The dried extracts were further used for phytochemical screening and antioxidant activities. Filtrates were evaporated to dryness using a rotary evaporator (Scilogex SCI100-S,USA) at 40°C (Akerele et al., 2008) .The residue obtained were dried and stored at 4°C until bioassayed (Mann et al., 2008; Nenaah & Ahmed, 2011). The yield percentage of the crude sample was determined by using the equation according to (Surendran et al., 2004).

Determination of Phytochemical Constituents

Phytochemical components of the leaf extracts such as saponins, tannins, alkaloids, phlobatanins and glycosides were determined according to the methods described by (Evans, 1989).

Free Radical Scavenging Activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

The different extracts of *Z. mauritiana* leaves were evaluated for 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. 4 ml of the extract stock solution was added to separate test tubes containing 1 ml DPPH solution at different concentrations from 20 to 100 µg/ml and mixed vigorously by hand. All the test tubes were kept at room temperature in a dark 45 min. The control was prepared following the same procedure without adding any crude extract. After incubation, the absorbance was measured at a fixed wavelength of 517 nm using UV-visible spectrophotometer (Jenway 6305, Staffordshire, UK) of all the concentrations of crude extracts (Öztürk et al., 2011) . The percentage of inhibition of each concentration of crude extract was calculated using the following formula:

DPPH radicals scavenged h (%) = $A_0 - A_1 / A_0 \times 100$

A_0 : Absorbance of the control

A_1 : Absorbance of the extract

Ascorbic acid was used as the standard.

Compounds Identification by Gas Chromatography-Mass Spectrometry

The identity of the phytochemicals for methanol, chloroform, ethyl acetate and hexane extracts were determined by gas chromatography-mass spectrometry (GC-MS) analysis using a Phenomenex, Torrance, GC-MS equipped with ZB-5mSi-fused silica capillary column (30 m x 0.25 mm, 0.50 µm). GC-MS had electron energy of 70 Ev, ion source temperature of 230 °C and electron emission of 34.6 µA. The temperature of analyzer was maintained at 150 °C. Helium was used as carrier gas at flow rate of 1ml/min. The injector and interface temperature was set at 290 °C and 360 °C, respectively. The oven temperature was programmed as 50 °C (1 min) to 310 °C (20 mins), at increasing rate of 6 ° C /min. Compounds were identified on the basis of comparison of their relative retention time and mass spectra with those of the NIST library data of GC/MS system (Ezhilan & Neelamegam, 2012).

Statistical Analysis

All test trials were carried out in triplicate. Mstatc computer software version 6.1 was used to perform analysis of variance (ANOVA) and to determine significant differences ($p<0.05$). Results were calculated and expressed as mean values and standard deviations (mean ± SD).

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RESULTS

Results of Leaves Extraction

The extract from the dried leaves of the plant were obtained using different solvents methanol, hexane, chloroform, ethyl acetate and water for three days each using maceration method. Table 1 shows the result of extraction in different solvent. The highest yield percentage was observed in aqueous extract, which is 6.70%, followed by chloroform (5.78 %), methanol (4.30 %), ethyl acetate (2.36 %) and then Hexane (1.04 %).

Table 1: Result of leaves extraction

Solvent	Dry Leaves (g)	Mass of Crude Extract (g)	Yields (%)
Hexane	500.01	5.22	1.04
Methanol	500.10	21.47	4.30
Ethyl acetate	500.05	11.81	2.36
Chloroform	500.00	28.92	5.78
Aqueous	500.08	33.47	6.70

Qualitative Phytochemical Screening

The present study revealed that the leaf extract of *Z. mauritiana* obtained by different solvents contained alkaloids, glycosides, flavonoids, phenols, tannins, steroids, saponins, terpenoids, phlobatannins, anthraquinones and resins (Table 2). All the phytochemicals were present in the aqueous extracts. Also, Phlobatannins were detected in all the extracts with the exception of hexane and ethyl acetate extracts. Steroids were also absent in methanol and ethyl acetate extracts. Anthraquinones were not detected in hexane, methanol, ethyl acetate and chloroform extracts respectively.

Quantitative Phytochemical Screening

Total phenolic content of *Z. mauritiana* leaves

The total phenolic content of the different leaf extracts of *Z. mauritiana* was evaluated by a modified FCR method with a gallic acid standard (Al-Saeedi & Hossain, 2015; Hossain et al., 2016) .The maximum amount of total phenols content in the leaf extracts was obtained in the chloroform extract and the minimum amount was obtained in the water extract. The extracts followed the order: Chloroform > methanol > hexane > ethyl acetate > Aqueous (Table 3).

Total flavonoids content of *Z. mauritiana* leaves

The total flavonoids contents of different polarity extracts of *Z. mauritiana* leaves were evaluated using a modified $AlCl_3$ method using quercetin as standard. The maximum amount of total flavonoids content in the leaf extracts were obtained in the methanol extract and the minimum amount was obtained in the ethyl acetate extract . The extracts followed the order: Methanol > chloroform > hexane > aqueous > ethyl acetate (Table 4).

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Table 2: The results of phytochemical screening of *Z. mauritiana* leaves

Organic compounds	Hexane	Methanol	Ethyl acetate	Chloroform	Aqueous
Alkaloids	+	+	+	+	+
Glycosides	+	+	+	+	+
Flavonoids	+	+	+	+	+
Phenols	+	+	+	+	+
Tannins	+	+	+	+	+
Steroids	+	-	-	+	+
Saponin	+	+	+	+	+
Terpenoids	+	+	+	+	+
Phlobatannins	-	+	-	+	+
Anthraquinones	-	-	-	-	+
Resins	+	+	+	+	+

Table 3: The results of Total phenolic content of *Z. mauritiana* leaves

Crude leaf extract	Total(µg /mg)
Aqueous	22.33 ± 0.34
Methanol	77.80 ± 1.12
Hexane	56.18 ± 1.44
Ethyl acetate	48.46 ± 0.67
Chloroform	84.60 ± 0.92

Results are expressed as µ g of gallic acid equivalent/mg of crude extracts ; Each value in the table is the mean (±) standard deviation (n = 3).

Table 4: The results of Total flavonoids content of *Z. mauritiana* leaves

Crude leaf extract	Total µg /mg
Aqueous	13.14 ± 0.34
Methanol	46.91±1.54
Hexane	18.05 ± 0.77
Ethyl acetate	11.71 ± 0.67
Chloroform	40.10 ± 3.90

Results are expressed as µ g of quercetin equivalent/mg of crude extracts; Each value in the table is the mean (±) standard deviation (n = 3).

The total flavonoids and phenolics contents of methanol, hexane and, chloroform extracts of *Z. mauritiana* leaves as shown in Table 3 and 4, revealed a significant (p<0.05) difference relative to the extraction solvents. Chloroform extract recorded the highest phenolics content of 84.60 ± 0.92 µg GAE/mg of extract, followed by methanolic extract recording 77.80 ± 1.12 µg GAE/mg of extract, then followed by hexane extract (56.18 ± 1.44 µg GAE/mg of extract), then ethyl acetate (48.46 ± 0.67 µg GAE/mg of extract) and then aqueous extract (22.33 ± 0.34 µg GAE/mg of extract) which showed the lowest content.

Antioxidant activity using DPPH method

From the four extracts investigated for antioxidant activity using DPPH method, the results were in the following order methanol > chloroform > hexane > ethyl acetate (Table 5). Also, the concentrations required to scavenge 50% of the DPPH radicals (IC_{50}) were investigated. The IC_{50} values of methanol, chloroform, hexane and ethyl acetate extracts were 37.62 µg/mL, 43.94 µg/mL, 45.16 µg/mL and 54.23 µg/mL, respectively. The IC_{50} value for ascorbic acid was 35.61 µg/ml (Figure 1). The results indicate that the antioxidant activity of the crude extracts of *Z. mauritiana* is higher than that of ascorbic acid.

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Table 5: DPPH radical scavenging activity (%) of different extracts of *Z. mauritiana*

Extract	Concentration of Samples (mg/ml)				
	20	**40**	**60**	**80**	**100**
AA	37.36±0.02	48.50±0.03	74.52±0.01	92.56±0.03	98.47±0.02
MLE	34.35±0.02	47.65±0.02	73.89±0.03	90.94±0.01	94.47±0.02
CLE	23.88±0.01	47.55±0.03	71.04±0.02	85.06±0.01	93.01±0.03
HLE	32.67±0.02	39.35±0.03	64.49±0.02	82.53±0.03	88.15±0.02
ELE	9.87±0.02	21.86±0.02	46.26±0.02	62.18±0.02	70.34±0.02

AA - Ascorbic acid
HLE - Hexane leaves extract
MLE - Methanol leaves extract
ELE - Ethyl acetate leaves extract
CLE - Chloroform Leaves extract

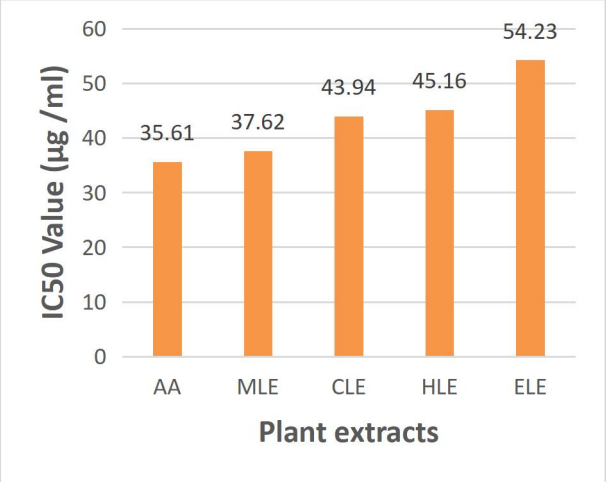

Figure 1: IC_{50} values of plant extracts

Phytocomponents Identified by GC-MS

GC-MS chromatogram analysis identified thirty -three phenolic compounds with different chemical structures from the different extracts of *Z. mauritiana* leaves (Table 6). The active compounds were established with their retention time (RT), molecular formula and the molecular weight (MW) with reference to previous study (Table 6). The major compounds identified in methanol extract were squalene (30.08%), n-hexadecanoic acid (28.90%) and *p*-coumaric acid (23.32%). Other important constituents identified were tetradecanoic acid (14.90%) and salviolinic acid (12.55%) in ethyl acetate extracts. On the other hand, a reasonable amount of α-linolenic acid (26.42%) and 17-betulinic acid (13.42%) were detected in hexane extracts. A considerable amount of palmitic acid (38.50 %) , stearic acid (15.78%) and methyl stearate (12.28%), were observed to be present in chloroform extracts.

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Table 6: Phytocomponents identified in the leaf extracts of *Z. mauritiana* by GC-MS

Solvent	Compounds	Retention time	% composition	Molecular Formula	Molecular weight(g/mol)
Methanol	4-O-caffeoylquinic acid	30.78	15.66 ± 0.30	$C_{16}H_{18}O_9$	354.31
	caffeic acid	18.34	4.93 ± 0.50	$C_9H_8O_4$	180.16
	Syringic acid	21.24	0.41 ± 0.12	$C_9H_{10}O_5$	198.17
	Epicatechin	28.98	12.46 ± 0.53	$C_{15}H_{14}O_6$	290.27
	p-coumaric acid	27.14	23.32± 0.90	$C_9H_8O_3$	164.16
	Quercitrin	29.30	13.44 ± 0.24	$C_{21}H_{20}O_{11}$	448.4
	Quercetin	17.89	4.84 ± 0.96	$C_{15}H_{10}O_7$	302.23
	Naringenin	31.77	1.11 ± 0.04	$C_{15}H_{12}O_5$	272.25
	Gallic acid	24.66	10.34 ± 0.76	$C_5H_6O_5$	170.12
	Squalene	40.20	30.08 ± 0.34	$C_{30}H_{50}$	410.7
	n- Hexadecanoic acid	26.30	28.90± 0.11	$C_{16}H_{32}O_2$	256.42
Ethyl acetate	Salviolinic acid	27.10	12.55 ± 0.60	$C_{26}H_{22}O_{10}$	494.4
	Luteolin	18.67	4.94 ± 0.70	$C_{15}H_{10}O_6$	286.24
	Cirsilineol	22.45	6.75 ± 0.80	$C_{18}H_{16}O_7$	344.3
	Tetradecanoic acid	28.73	14.90± 0.11	$C_{14}H_{28}O_2$	228.37
	Hyperoside	4.80	0.30 ± 0.01	$C_{21}H_{20}O_{12}$	464.4
	Phthalic acid, butyl undecyl ester	15.90	7.31 ± 0.01	$C_{23}H_{36}O_4$	376.5
	Z-2-Dodecenol	13.30	8.26 ± 0.01	$C_{12}H_{24}O$	184.32
	L-(+)-Ascorbic acid 2,6-dihexadecanoate	27.12	3.24 ± 0.14	$C_{38}H_{68}O_8$	652.9
Hexane	Myristic acid	20.33	0.70 ± 0.51	$C_{14}H_{28}O_2$	228.37
	Ferulic acid	45.60	2.71± 0.44	$C_6H_{10}O_4$	194.18
	Betulinic acid	52.10	13.42 ± 0.02	$C_{30}H_{48}O_3$	456.7
	D-Allose	45.90	1.72± 0.02	$C_6H_{12}O_6$	180.16
	Linoleic acid	31.72	1.35 ± 0.05	$C_{18}H_{32}O_2$	280.4
	α-Linolenic acid	30.81	26.42± 0.01	$C_{18}H_{30}O_2$	278.43
Chloroform	Methyl palmitate	26.50	2.80 ± 0.24	$C_{17}H_{34}O_2$	270.5
	Palmitic acid	27.12	38.50 ± 0.06	$C_{16}H_{32}O_2$	256.42
	Rutin	27.28	3.22 ± 0.11	$C_{27}H_{30}O_{16}$	610.5
	Methyl stearate	30.11	12.28± 0.05	$C_{19}H_{38}O_2$	298.5
	Stearic acid	31.19	15.78 ± 0.25	$C_{18}H_{36}O_2$	284.5
	Bacchotricuneatin C	34.11	3.45 ± 0.14	$C_{20}H_{22}O_5$	342.4
	Lauric acid	17.03	1.66 ± 0.05	$C_{12}H_{24}O_2$	200.32
	Vitamin E	44.11	5.38 ± 0.20	$C_{29}H_{50}O_2$	430.7

DISCUSSION

Phytochemical screening results of different solvent extracts of Z. *mauritiana* leaves suggests that the extracts of *Z. mauritiana* contains phytochemical compounds (Table 2).The results confirmed the presence of various health-enhancing phytochemical constituents, including tannins, steroids, phenolic compounds, alkaloids, saponins, and flavonoids. The presence of these secondary metabolites suggests that this plant has some medicinal properties. The results of current study is in agreement with previous studies, (Najafi, 2013) which showed that the leaves of *Z. mauritiana* comprises phenolic compounds, saponins, and tannins. Results of the study by (Parmar et al., n.d.) also showed that leaves of *Z. mauritiana* possess saponins, phenolic compounds, lignins, tannins, and glycosides. The phenolics were known to possess various biological activities that include anticarcinogenic,antimicrobial,

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antimutagenic, antidiabetic, antioxidative, antiallergic and anti-inflammatory activities (Arts & Hollman, 2005) .Steroids are associated with antimicrobial and insecticidal properties as well as treatment for heart muscles contraction. Similar to phenolics, tannins were found to be linked with general antioxidant and antimicrobial activities (Mainasara et al., 2012) . Very importantly, saponin of *Z. mauritiana* were reported to be used in treatment of hyperglycemia, weight loss and hypercholesterolemia, and were also established to confer anticancer, antioxidants, antimicrobial and anti-inflammatory activities (De-Lucca et al., 2005) . It is noteworthy to mention here that, the presence as well as the absence of certain compounds from the plant extracts could be due to the impact of different solvents and content of secondary metabolites (Bettaieb Rebey et al., 2012).

Phenolic acids and flavonoids are considered to be an important group of potent natural antioxidants (Duthie & Morrice, 2012). These compounds have broad-spectrum of biological activities and could decrease occurrence rate of infectious diseases and disorders due to oxidative stress (Huang et al., 2009) . These findings are in consistence with those reported in a previous work that described the extraction efficacy of solvents (chloroform > methanol > hexane) in identifying phenolic compounds from *Hibiscus cannabinus* L. seeds (Yusri et al., 2012). However, this study reported lower total flavonoids content of 46.91±1.54 µg QE/mg of extract in methanol followed by 40.10 ± 3.90 µg QE/mg of extract in chloroform and then 18.05 ± 0.77 µg QE/mg of extract in hexane.

The polarity of the extraction solvents with respect to solubility of phytocompounds is responsible for the variation in concentration of the phenolics and flavonoids in the extracts (Bae et al., 2012) . At cellular level, the phenolic compounds are mostly found in the vacuoles of colored tissues such as flower petals or leaves (Vermerris & Nicholson, 2007) . Additionally, others studies indicated that the flavonoids present in the epidermis or cuticula of the leaves, occur as the lipophilic flavonoids, demonstrated to be among the most active phenolic compounds that mediate plant resistance against wide range of crop antagonists (Pomilio et al., 1992; Wilfred & Nicholson, 2006)

In the present study, DPPH free radicals scavenging potential of different solvents (methanol, chloroform,ethyl acetate and hexane) extract of *Z. mauritiana* leaves were assessed. DPPH free radicals scavenging of methanol extract of *Z. mauritiana* leaves showed significant radical scavenging activity with increasing concentration of the extracts (Table 5). This is consistent with a previous work in which the proportion of free radicals scavenging was found to be concentration dependent (Heo et al., 2007) . The higher absorbance reveals greater reducing power of the plant. Concentration-dependent reducing power was observed in methanolic leaf extract of *Z. mauritiana*. The highest protection of 94.47% was observed in 100 mg/ml. The entire order of free radical scavenging activity of the solvent extracts used are as follows: methanol > chloroform > hexane > ethyl acetate (Fig. 1). The DPPH free radical-scavenging activity results indicate that components within the extracts are capable of scavenging free radicals through hydrogen-electron donation mechanisms.Therefore, it should be able to prevent the initiation of damaging free radical-mediated chain reactions in susceptible matrices, such as biological membranes. Furthermore, this suggests the extracts ability to scavenge different free radicals in different systems. Consistently, this suggests their potential use as therapeutic agents for treating radical-related pathological damage and may provide health benefits to the consumers. In general,

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the result was in conformity with the antioxidant activity value of organic crude extracts of *Ziziphus. jujuba* as reported by (Al-Reza et al., 2009) . DPPH scavenging activity presented by IC_{50} value as presented in Fig 1 shows that methanol extract has higher antioxidant activity. The lower the IC_{50} value, the higher the antioxidant activity (Kedare & Singh, 2011).

CONCLUSION

The crude extracts of *Z. mauritiana* leaves are prolific in phytochemical constituents with considerable level of antioxidants activities. Additionally isolation and purification of these phytochemical constituents may yield more significant levels of antioxidant activity. Thus, it can serve as new drugs and can be screened for the treatment of many diseases and disorders.

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