QUANTITATIVE PHYTOCHEMEMICAL DETERMINATION AND ANTIOXIDANT STUDY OF DIOSPYROS *Mespiliformis* (Jackalberry) LEAVES EXTRACT IN RATS

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ABSTRACT

Medicinal plants are known to contain phytochemicals which are responsible for their healing potentials. *Diospyros mespiliformis* (*D. mespiliformis*) is a herbal plant used locally in Nigeria for the treatment of many ailments including, diabetes, high blood pressure, coronary heart diseases, ulcer, etc. The quantitative phytochemical constituents as well as the *in vitro* and *in vivo* antioxidant properties of the methanol extract of *D. mespiliformis* leaves were determined. The quantitative phytochemicals estimation of the leaf extract was carried out using standard procedures. The *in vitro* antioxidant potential was assayed by the 1,1-diphenyl-2-picryhydrazyl (DPPH) method using ascorbic acid as standard antioxidant drug, while the *in vivo* antioxidant activity was carried out by determining the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and lipid peroxidase (LP). Oxidative stress in rats was induced with 70% ethanol. Total alkaloids, tannins and total phenols were found in approximately equal amounts (0.06%). Total flavonoid was found to be the highest in concentration (0.11%) while total saponin was the lowest (0.01%). The DPPH *in vitro* free radical scavenging assay showed that the inhibition concentration (IC_{50}) of the extract (17.10 μg/ml) was lower than that of ascorbic acid, (21.96 μg/ml) making it a better antioxidant. The extract at 200 and 400 mg/kgbw increased the SOD activity ($0.30\pm0.03$and 0.48 ± 0.02 U/L) in a dose dependent manner and were found to be significantly different when compared to the negative control (oxidative stress induced, not treated) (0.19 ± 0.02 U/L). Catalase activity was significantly ($p<0.05$) lower for both the 200 & 400 mg/kgbw *D. mespiliformis* treatment concentrations, which were $0.07\pm$ 0.01 and $0.03\pm$ 0.01 U/L respectively when compared with the negative control ($0.14\pm$ 0.02 U/L). Lipid peroxidation level were 5.11 ± 0.10, and 4.28 ± 0.69 nmol/mg protein for 200 & 400 mg/kgbw *D. mespiliformis* treatment concentrations respectively, the activities decreased significantly when compared with the negative control (9.11 ± 0.1 nmol/mg protein). It can therefore be suggested that *D. mespiliformis* may be used as antioxidant due to its free radical scavenging potential.

Keywords: *Diospyros mespiliformis*, Phytochemicals, Antioxidant, Ethanol.

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INTRODUCTION

Oxidative stress has been implicated in the etiology of many serious diseases such as diabetes, high blood pressure, coronary heart disease, ulcers etc. Medicinal plants contain diverse phytochemicals in varying amounts and these phytochemicals have been reported to have potent antioxidant effect with

little or no side effects (Bashir *et al.*, 2016).
Ethanol is reported to cause disturbances in gastric secretion, damage to the mucosa, alterations in the permeability, gastric mucus depletion and free radical production. This is attributed to the release of superoxide anion and hydroperoxy free radicals during metabolism of ethanol (Bech *et al*, 2009).
Herbal medicine is fast emerging as an alternative treatment to available synthetic drugs for treatment of stress related illnesses possibly due to lower costs, easier availability, and fewer adverse effects. Many tropical herbs have been scientifically reported to possess potent antioxidant activity (Kumar, 2011; Singh *et al.*, 2008).
Diospyros mespiliformis is commonly called Jackal-berry or African ebony, In Nigeria it is known in Hausa as Kanya and in Yoruba as Igidudu. It is found in Savanna 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 (Bala, 2006). The plant flowers in the months of April and May (Normandin, 2007). The plant is reputed for its medicinal values, and is used in ethno medical practice for treating various ailments that include sleeping sickness, malaria, headache, cough, leprosy, helminth infection (Belemtougri *et al.*, 2006) toothache and (Adzu *et al.*, 2015; Bagalkotkar *et al.*, 2011). Its seeds are also known to have nutraceutical value in managing high cholesterol, reducing risk of type-2 diabetes, and for weight control (Chivandi and Erlwanger, 2011). Useful biologically active compounds isolated and identified from the plant includes naphthoquinone epoxide, α-amyrin, β-sitosterol, betulin and betulinic acid amongst others (Mohammed *et al.*, 2009). This study aimed at determining quantitatively the phytochemicals and the antioxidant effect of *D. mespiliformis* leaves extract in rats.

MATERIALS AND METHODS

Collection of *D. mespiliformis*

Fresh leaves of *Diospyros mespiliformis* was obtained from Agwara Local Government Area, of Niger State, Nigeria. Taxonomic authentications of the plant was carried out at the Department of Biological Sciences, Federal University of Technology, Minna, Niger State.

Preparation of crude extract

The fresh leaves of *Diospyros mespiliformis* collected were destalked, washed with clean-water, dried at room temperature (27 C) and grounded using a grinder mill. The powdered leaves (300 g) was extracted with 80% methanol (600 ml) using the continuous cold extraction method for 72 hours (Kalra *et al.*, 2011). The extract was filtered, concentrated using a rotary evaporator and the resulting extract was concentrated in a water bath and stored in refrigerator prior to use.

Quantitative phytochemicals determination

Quantitative concentration of alkaloid, tannins, flavonoids, saponins, phenol were estimated using the method described by Harborne, (1976), (AOAC, 1999).

Antioxidant activity of *D. mespiliformis* extract

DPPH radical scavenging assay

DPPH (1,1-Diphenyl-2-Picrythydrazyl) scavenging activity was measured by the spectrometric method as described by Tsado *et al.* (2016). The principle of this assay was based on the free radicals

DPPH having characteristic absorption at 517 nm which decreased significantly when exposed to antioxidant by receiving hydrogen atom or electron. The free radical scavenging activity was calculated using the formula below:

$$\% \text{ Inhibition} = \frac{AB - AE}{AE} \times 100$$

Where

AB= absorbance of blank sample

AE= absorbance of extract

Experimental Animals

Healthy albino rats of average weight 150-180g were purchased from Biochemistry Department, Federal University of Technology, Minna, Niger State Nigeria. The rats were kept in clean plastic cages and maintained under standard laboratory conditions in the Biochemistry laboratory. They were allowed unrestricted access to poultry feed and water *ad-libitum*. The study was carried out according to the Guide for the Care and the Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, USA.

Experimental Design

Twelve normal healthy Wister albino rats were divided into three groups of 3 animals each. Group 1 consists of untreated control rats. Group 2 served as the positive control while group 3 and 4 received 200 and 400 mg/kgbw of *D. mespiliformis* leaves extract respectively.

Doses and Route of Administration

Ethanol (70 %) was administered at a dose of 0.5 mL/100 g-body weight on 24 hour empty stomach to induce oxidative stress. *D. mespiliformis* leaves extract were orally given at doses of 200 and 400 mg/kg body weight daily for a week (Gokhale *et al.,* 2003). All animals were deprived of food (but not water) for 24 hours prior to being subjected to ulcerogenic compound (ethanol 0.5 ml/100g body weight) (Suleyman *et al.,* 2004; Vasudeva *et al.,* 2012).

Preparation of Tissues and Plasma Samples

Gastric tissue samples were homogenized by using sucrose solution after the measurement of ulcer index. Centrifugation (3000 rpm) of homogenized tissues and collection of supernatants were carried out. These supernatants were stored under refrigeration for further estimation of various biochemical markers. Blood samples were collected in anticoagulant bottles and centrifuged at 3000 rpm. The supernatants were collected and stored under refrigeration for further estimation of various biochemical markers.

Superoxide dismutase (SOD) is an enzyme responsible for the removal of superoxide formed from oxygen reactions in tissues. This was done by the method of Winterbourn *et al.,* (1975). The ability of superoxide dismutase (SOD) to inhibit the antioxidation of epinephrine at pH 10.2 forms the basis for a simple assay of dismutase. Oxygen generated from xanthine oxidase reaction causes the oxidation of epinephrine to adrenochrome produced per O_2 introduced increased with increasing pH, and with increasing concentration of epinephrine.

A 10 times diluted tissue supernatant, 0.05M phosphate buffer (pH 7.8) and 0.3M adrenaline were employed to monitored the increase in absorbance at 480 nm every 30 seconds for 150 seconds.

Calculation:

Increase in absorbance per minute = $\underline{A_3 - A_0}$

2.5

Where
Ao = absorbance after 30 seconds
A3 = absorbance after 150 seconds

Estimation of Catalase Activity (CAT)
This was carried out by the method described by Aebi, (1984). A standard curve of hydrogen peroxide was first prepared by dispensing various volumes (0.00, 0.05, 0.10, 0.15, 0.30 e.t.c) of H_2O_2 into test tubes and 2 ml of dichromate/acetic acid was added to each. It was heated for ten minutes in a boiling water bath after cooling the volume of the reaction mixture was made up to 3 ml with distilled water and absorbance was read at 570 nm on a spectrophotometer the H_2O_2 content was extrapolated from the standard curve.

Estimation of Lipid Peroxidation in Tissue
Thiobarbituric acid reactive substance (TBARS) in the tissue was estimated using the method of Fraga *et al.*, (1988). The formation of malondialdehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. A low pH of 2-3 and high temperature (100^{0}C), malondialdehyde (MDA) binds thiobarbituric acid (TBA) to form a pink complex which absorbs maximally at 532nm.

Data Analysis
Values were analyzed using statistical package for social science (SPSS) version 16 and presented as means ± SE of the mean. Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The level of significance was set at $p < 0.05$ (Adamu and Johnson, 1997)

RESULTS

Quantitative phytochemical Analysis.
Table 2 showed the quantitative phytochemical compositions of methanol leaves extract of *Diospyros mespiliformis*. The extract was found to contain total phenol, total alkaloids and total tanins in approximately equal amount (0.06%) while, 0.11 %, 0.01 % were determined for total flavonoids, and of Saponins respectively.

***In vitro* Antioxidant Study**
The results of DPPH (1,1-Diphenyl-2-Picrythydrazyl) radical scavenging activity of methanol leaf extract of *D. mespiliformis* are presented in Table 3 The extracts and the standard antioxidant (Vitamin C) promoted an inhibition of DPPH radical with increasing concentrations. DPPH radical scavenging activity of methanol leaf extract of *D. mespiliformis* ranged from 24.10 (at 5 ug/ml) – 67.22 % (at 25 ug/ml). The IC_{50} (concentration that inhibits 50 % of the DPPH radical) values of *D. mespiliformis* and ascorbic acid were 17.10 and 21.96 μg/mL respectively.

***In vivo* antioxidant study**
The levels of Malondialdehyde (MDA) as an index of lipid peroxidation activity was significantly ($p< 0.05$) higher in all experimental group when compared with the negative control group. Administration of the methanol extract of *Diospyros mespiliformis* leaves at dose of 400 mg/kg (Curative) caused significant ($p < 0.05$) reduction in the levels of Malondialdehyde (MDA) when compared with positive control. However, the group of rats treated with 200 mg/kg of the extract compared well with the positive control. Similarly, SOD activities were significantly ($p< 0.05$) lower in all

experimental groups when compared with the negative control group. Catalase activities were significantly ($p < 0.05$) high in positive control when compared with negative control group.

Table 1: Quantitative phytochemical compositions of 80% methanol leave extract of *Diospyros mespiliformis*.

Phytochemicals	Concentration (mg/100g)	Percentage concentration
Total phenol	59.32±0.86	0.06 ± 0.001
Total flavonoids	114.44±3.43	0.11± 0.003
Tannins	56.91±0.97	0.06± 0.001
Alkaloids	61.18±0.54	0.06± 0.001
Saponins	9.47±1.59	0.01±0.002

Values are mean±SEM of triplicate determinations.

Table 2: DPPH (1,1-Diphenyl-2-Picrythydrazyl) free radical scavenging activity of methanol extract of *D. mespiliformis* leaves in rats.

Concentration ug/ml	*D. mespiliformis* (% inhibition)	Ascorbic acid (% inhibition)
5	24.10±0.13	4.615±0.16
10	32.31±0.19	18.04±0.14
15	34.65±0.23	29.49±0.32
20	66.41±0.17	39.03±0.47
25	67.22±0.13	62.97±0.34
*IC_{50}	17.10μg/mL	21.96μg/mL

Values are mean±SEM of triplicate determinations.
*IC_{50} values were extrapolated from a plot of % inhibition against concentration (ug/ml), see graphs below.

Figure 3.1: DPPH Free Radical Scavenging Curve for Ascorbic Acid.

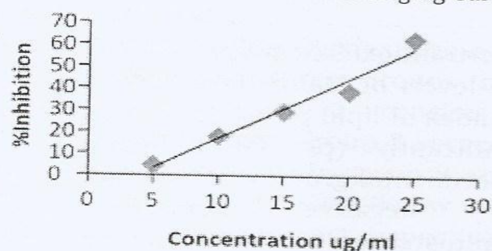

Figure 3.2: DPPH Free Radical Scavenging Curve for *D. mespiliformis* Leaves Extract.

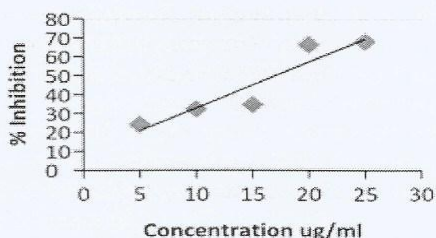

Table 3: *In vivo* effect of methanol extract (curative) of *D. mespiliformis* leaf on lipid peroxidation and antioxidant enzymes in ethanol induced oxidative stress in rats.

Groups	Superoxide dismutase (SOD) (U/L)	Catalase (U/L)	Lipid Peroxidation (LP) MDA(nmol/mg protein)
Negative control	0.60 ± 0.03^{c}	0.09 ± 0.01^{ab}	3.420 ± 1.07^{a}
Positive control	0.19 ± 0.02^{a}	0.14 ± 0.02^{b}	9.11 ± 0.10^{c}
Extract 200 mg/kgbw	0.30 ± 0.03^{b}	0.07 ± 0.01^{ab}	5.11 ± 0.10^{b}
Exract 400 mg/kgbw	0.48 ± 0.02^{b}	0.03 ± 0.01^{a}	4.28 ± 0.69^{a}

Values are mean ± SEM of 3 determinations. Values along the same column with different superscripts are significantly different ($p < 0.05$).

DISCUSSION

The derived values for the quantitative phytochemical determinations for flavonoids, phenol, tannin, alkaloids and saponins agrees with the report of Oguche and Nzelibe (2016). The presence of flavonoid (0.11%) a phytochemical with antioxidant properties suggest the reason why *Diospyros mespiliformis* plant is used for the prevention of stress mediated cell injury which was also reported by Musonda and Clipman, (1998).
The DPPH free radical inhibition concentration (IC_{50}) value (17.10 µg/mL) recorded for *Diospyros mespiliformis* leaves extract during the *in vitro* antioxidant study as compared to that of ascorbic acid standard (21.96 µg/mL) implies that the extract has a better free radical scavenging activity than the ascorbic acid.
This could be as a result of the presence of the antioxidant phytochemical such as flavonoid which had been reported to be associated with free radical scavenging activities. The free radical scavenging properties demonstrated by *Diospyros mespiliformis* leaves extract is supported by the report of Oulare *et al.* (2015) who found out that the significant amount of polyphenols in *Diospyros mespiliformis* plant can provide protection against development and progression of many chronic pathological conditions like cancer, diabetes, cardiovascular problems and aging.
The significant ($p< 0.05$) alteration in the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and lipid peroxidase (LP)) in the positive control group as compared to the normal control were in agreement with the report of Pallavi and Balaraman (2011). This could be due to the production of reactive oxygen species that was brought about by ethanol. *In vivo* antioxidant enzymes (SOD and CAT) in an effort to protect the cells are depleted, as such, their levels in the tissue is lowered.

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