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In vitro Antioxidant Activities of Ethanol, Ethyl Acetate and n-Hexane Extracts of *Mangifera indica* Leaves

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Abstract

Mangifera indica leaves have been in use in folk medicine since ancient time, for the treatment and/or management of numerous health complications. In this study, total phenols and flavonoids contents, as well as *in vitro* antioxidant activities of ethanol, ethyl acetate and n-hexane extracts of *M. indica* leaves were determined and compared. The *in vitro* antioxidant activities were determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power, and inhibition of lipid peroxidation assays, respectively. The total phenols were found to be present in the descending order of; ethanol extract > ethyl acetate extract > n-hexane extract, while flavonoids were observed in the descending order of; n-hexane extract > ethanol extract > ethyl acetate extract. The maximum percentage DPPH radical scavenged (at 100 µg/mL), ferric reducing power (at 400 µg/mL) and inhibitory activity on lipid peroxidation (at 400 µg/mL) exhibited by the ethanol extract (94.40%, 88.73%, and 84.05%, respectively) were significantly greater ($p < 0.05$) than the ethyl acetate extract (79.46%, 68.74%, and 79.73%, respectively at 400 µg/mL) as well as n-hexane extract (45.30%, 58.79%, and 67.78%, respectively at 100 and 400 µg/mL). It can be concluded that the extracts possess anti-oxidant activities and may be employed in the treatment and/or management of oxidative stress related diseases.

Keywords: *Mangifera indica*, phenolics, flavonoids, antioxidant, DPPH radical.

Introduction

The search for compounds of natural origins that possess antioxidant activity has recently increased because of their pharmacological values in treating and/or managing numerous chronic diseases including cancer, cardiovascular diseases, among others. About two-thirds of standard drugs are derived from plants (Patridge et al. 2016). There are many studies in support of the fact that oxidative stress is implicated in numerous diseases of humans and animals. Free radicals are naturally produced in the human bodies. Compounds that quench the actions of free radicals are called anti-oxidants. When there is overload of free radicals than the body can control, these free radicals cause the oxidation of vital biomolecules, such biomolecules as lipids, proteins and deoxyribonucleic acid (DNA) in healthy human cells. As reported by Naidu et al. (2016), this oxidation of biomolecules

leads to stimulation of carcinogenesis, cardiovascular disease, atherosclerosis, aging and inflammatory diseases.

Antioxidants can be classified into two classes, namely natural and synthetic antioxidants. Natural antioxidants have been employed in fields owing to their safe nature than the synthetic antioxidants. Hossain et al. (2011) reported that natural antioxidants are often used in food industries where they are added to foods for preservation due to their inhibitory effects on the radical chain reactions of oxidation by inhibiting the initiation and elongation step resulting in reactions termination and a delayed oxidation process. Enzymes are inactivated and vital cellular constituents are damaged by reactive oxygen species such as superoxide anion (O_2^-), hydroxyl radicals (OH^-) and nitric oxide (NO^-) leading to injury. Thus, compounds attributed with antioxidant properties may be of use in the treatments of many diseases (Parim et al. 2015).

Mango leaf has been found to possess numerous pharmacologic benefits owing to its phytochemicals composition (Burton-Freeman et al. 2017). There exist numerous literatures recommending mango leaves, bark, seeds, flowers and raw and ripe fruits for multitudinous pharmacological and medicinal benefits (Shah et al. 2010, Gupta and Jain 2014, Stohs et al. 2018, Burton-Freeman et al. 2017, Imran et al. 2017). In addition, many research studies indicate that mango leaves and fruit possess antioxidant, anti-inflammatory, gastroprotective (Stohs et al. 2018), anti-diabetic, cardioprotective, hypolipidemic (Imran et al. 2017), anti-tumour, wound healing, anti-pyretic, anti-bacterial, anti-spasmodic, anti-carcinogenic, anti-viral, hepatoprotective, immunomodulatory, and anti-dysentery properties (Shah et al. 2010, Gupta and Jain, 2014, Burton-Freeman et al. 2017). Although, *in vitro* antioxidant activities of *M. indica* leaves have been carried out mostly by using its polar solvent extracts such as aqueous and ethanol extracts. Nevertheless, little or no research has been carried out using polar, mid polar and non-polar solvents extracts of this plant leaves. Hence, this study was undertaken to determine and compare the *in vitro* antioxidant activities of ethanol, ethyl acetate and n-hexane extracts of *M. indica* leaves.

Materials and Methods

Materials

Plant collection

Fresh leaves of *M. indica* were collected from FUTMIN ventures, Federal University of Technology, Minna, Nigeria on 25th April, 2020. The plant material was identified at the department of Plant Biology, Federal University of Technology, Minna, Nigeria with voucher number FUT.MIN/SLS/PB-020-019 and the specimen was deposited at herbarium unit of the university.

Chemicals and reagents

The chemicals and reagents used in this study were of analytical grade, and products of Sigma Aldrich. The chemicals and reagents used included methanol, distilled water, phosphate buffer, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium hexacyanoferrate(III), ferric chloride, thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), ferrous sulphate, trichloroacetic acid (TCA), to mention just a few.

Methods

Sample preparation and extraction

The collected fresh leaves were rinsed with distilled water and air-dried at room temperature (35 °C) for a period of two (2) weeks. After the leaves had dried, they were pulverised using electric blender (Eurosonic, ES-242). Thereafter, one hundred grams (100 g) of the powdered sample was extracted successively with n-hexane, ethyl acetate and ethanol, respectively in order of increasing polarity using Soxhlet extractor. The extracts were collected in separate clean beakers after extraction and concentrated on water bath at

70 °C. The concentrated extracts were kept in refrigerator at 4 °C until further use.

Determination of phenolics content of the extracts: determination of total phenols

The method of Singleton et al. (1999) was used to determine total phenol content of extracts. Briefly, 0.01 g of each extract was dissolved in 10 mL of distilled water, and 0.5 mL was oxidized by adding 2.5 mL of 10% Folin-Ciocalteu's reagent which was then neutralized by 2 mL of 7.5% sodium carbonate. The reaction mixture was incubated at 45 °C for 40 minutes. Absorbance was read at 765 nm using double beam Shimadzu UV spectrophotometer, UV-1800. Gallic acid was used to prepare the calibration curve.

Flavonoids determination

Flavonoids content of the extracts was determined using the method of Chang et al. (2002). In this method, 0.01 g of each extract was weighed and dissolved in 10 mL of distilled water. Then 0.5 mL of each extract was added to a test tube containing 1.5 mL of absolute methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water and incubated at ambient temperature for 30 minutes. The absorbance was read at 415 nm with double beam Shimadzu UV-spectrophotometer, UV-1800. Quercetin was used to prepare the calibration curve.

***In vitro* antioxidant assays**

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The antioxidant activities of the plant extracts were estimated using the DPPH radical scavenging assay as described by Oyaizu (1986). Different concentrations of extracts and ascorbic acid (control) (50, 100, 200 and 400 µg/mL) were prepared from stock solutions (1000 µg/mL), prepared by weighing and dissolving 0.01 g of the extracts and ascorbic acid, respectively in 10 mL of methanol. Thereafter, 2 mL of 0.004% DPPH in methanol was added to 1 mL of various concentrations of plant extracts and ascorbic acid, respectively. The reaction mixtures were incubated at 25 °C for 30 minutes. The absorbance of each test mixture was read against blank at 517 nm using double beam Shimadzu UV-1800 series spectrophotometer. The experiment was performed in triplicates. The percentage antioxidant activity was calculated using the formula below:

$$\% \text{ Inhibition} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}}\ x\ 100$$

Where; Ablank = Absorbance of control, Asample = Absorbance of the sample.

FRAP (ferric reducing antioxidant power) assay

Estimation of antioxidant activities of the plant extracts via ferric reducing antioxidant power assay were conducted according to the method of Oyaizu (1986). Stock solutions of plant extracts and ascorbic acid (control) (1000 µg/mL) were prepared, from which different concentrations of 50, 100, 200 and 400 µg/mL were prepared. In this assay, 1 mL of each plant extracts and ascorbic acid concentration was mixed with 1 mL of 0.2 M sodium phosphate buffer and 1 mL of 1% potassium hexacyanoferrate(III), respectively. The reaction mixtures were incubated at 50 °C for 20 minutes. Thereafter, 1 mL of 10% TCA was added. The reaction mixtures were then centrifuged at 3000 rpm for 10 minutes at room temperature. Then 1 mL of each supernatant obtained was mixed with 1 mL of distilled water and then 0.2 mL of 0.1% ferric chloride was added. The blank was prepared in the same way as samples except that the extracts were replaced by distilled water. The absorbance of the test mixtures was read at 700 nm. The percentage antioxidant activity was calculated using the formula below.

$$\% \text{ activity} = \frac{Asample - Ablank}{Asample}\ x\ 100$$

Inhibition of lipid peroxidation

Using the method of Halliwell et al (1995), the inhibitory effects of the extracts on lipid peroxidation were determined via thiobarbituric acid reactive substances (TBARS) assay with slight modification. Briefly, 0.5 mL of 10% egg homogenate was added to 0.1 mL of extract or ascorbic acid (control) at various concentrations (50, 100, 200 and 400 µg/mL) and made up to 1 mL with distilled water. Afterward, 0.05 mL of $FeSO_4$ was added to the mixtures and incubated for 30 minutes. Then, 1.5 mL of acetic acid and thiobarbituric acid in sodium dodecyl sulphate were added, respectively. The resulting reaction mixture was vortexed and incubated at 95 °C for 1 hour. The reaction mixtures were cooled and 5 mL of butanol was added to each of the reaction mixtures and centrifuged at 1200 x g for 10 minutes and the absorbance of the upper organic layer was read at 532 nm. The percentage inhibition of lipid peroxidation was calculated using the formula below.

$$\% \text{ Inhibition} = \frac{Ablank - Asample}{Ablank}\ x\ 100$$

Statistical analysis

The data were analyzed by One-way Analysis of Variance (ANOVA) using Statistical Product and Service Solutions (SPSS). The results were expressed as mean ± SD (standard deviation). The differences in means among the extracts were compared using "Duncan multiple range test". A *p*-value less than 0.05 was considered significant ($p < 0.05$).

Results

Estimation of phenolics compositions of the extracts

Table 1 shows the phenolic contents of the extracts, wherein, the phenols concentrations (mg/g) were observed in descending order of; ethanol extract > ethyl acetate extract > n-hexane extract (126.43 > 112.65 > 52.16 mg/g, respectively), and flavonoids concentrations (mg/g) were found to be in descending order of; n-hexane extract > ethanol extract > ethyl acetate extract (40.48 > 28.44 > 20.65 mg/g, respectively).

Table 1: Phenolics compositions of the extracts

	Phenols (mg/g)	Flavonoids (mg/g)
Ethanol extract	126.43 ± 0.51^{c}	28.44 ± 0.24^{b}
Ethyl acetate extracts	112.65 ± 0.33^{b}	20.65 ± 0.32^{a}
n-Hexane extract	52.16 ± 0.48^{a}	40.84 ± 0.29^{c}

Values are presented as mean ± standard deviation of triplicates. Values with different superscripts in the same column are significantly different at $p < 0.05$.

DPPH radical scavenging activities of the extracts

The percentage DPPH radical scavenging activities of the extracts are shown in Figure 1. The activities of the extracts as well as ascorbic acid were found not to be dose dependent. The maximum percentage DPPH scavenged was observed at 100 µg/mL for the ascorbic acid, ethanol and n-hexane extracts, while it was observed at 400 µg/mL for the ethyl acetate extract. However, the maximum percentage DPPH radical scavenged by the ethanol extract (94.40%) was significantly higher ($p < 0.05$) than those of the n-hexane extract (45.30%) and ethyl acetate extract (79.46%), but comparable ($p > 0.05$) to that of ascorbic acid (94.72%).

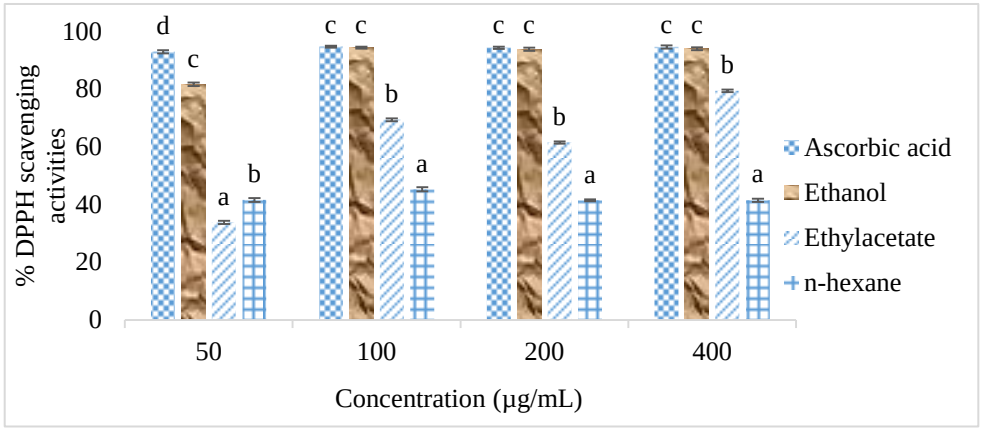

Figure 1: Percentage DPPH radical scavenging activities of ethanol, ethyl acetate and n-hexane extracts of *M. indica* leaves. Values are presented as mean ± standard deviation of triplicates. Values with different superscripts at the same concentration are significantly different at $p < 0.05$.

Ferric reducing antioxidant power of the extracts

Figure 2 shows the percentage ferric reducing powers of the extracts. A concentration-dependent activity was shown by ethanol and ethyl acetate extracts as well as ascorbic acid, respectively, whereas the activity of the n-hexane extract was found not to be dose dependent. For the ethanol extract, ethyl acetate extract and ascorbic acid, the maximum percentage ferric reducing power was obtained at concentration of 400 µg/mL, while it was obtained at concentration of 100 µg/mL for the n-hexane extract. The percentage ferric reducing power exhibited by ethanol extract (88.73%) was observed to be significantly higher ($p < 0.05$) than the ones exhibited by the ethyl acetate extract (68.74%) and n-hexane extract (58.79%), but non-significantly different ($p > 0.05$) from that of ascorbic acid (88.54%).

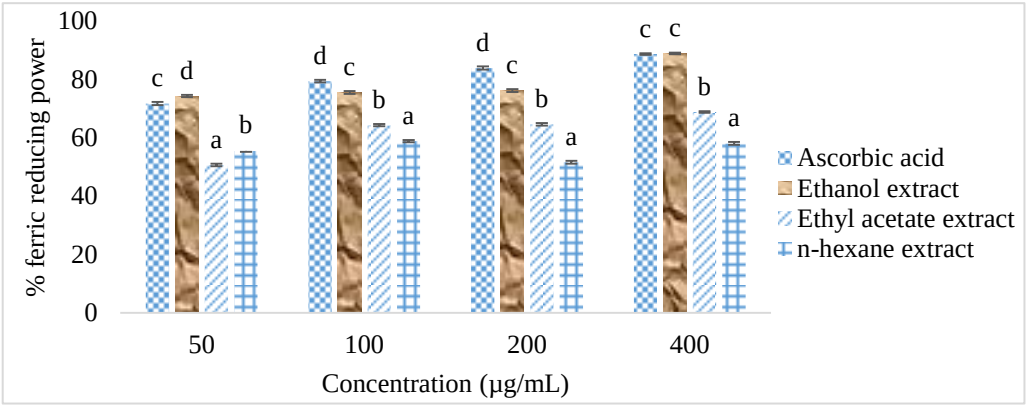

Figure 2: Percentage ferric reducing powers of the ethanol, ethyl acetate and n-hexane extracts of *M. indica* leaves. Values are presented as mean ± standard deviation of triplicates. Values with different superscripts at the same concentration are significantly different at $p < 0.05$.

Inhibition of lipid peroxidation by the extracts

The inhibitory activities of the extracts and ascorbic acid on lipid peroxidation were observed not to be concentration dependent, although, the maximum percentage inhibitory activity was observed at 400 µg/mL for the extracts and ascorbic acid. The Percentage inhibitory activity (84.05%) exhibited by the ethanol extract on lipid peroxidation was found to be significantly greater ($p < 0.05$) than those of ethyl acetate extract (79.73%) and n-hexane extract (67.78%), however, this was comparable ($p > 0.05$) to the percentage inhibitory activity of ascorbic acid (83.17%) as depicted in Figure 3.

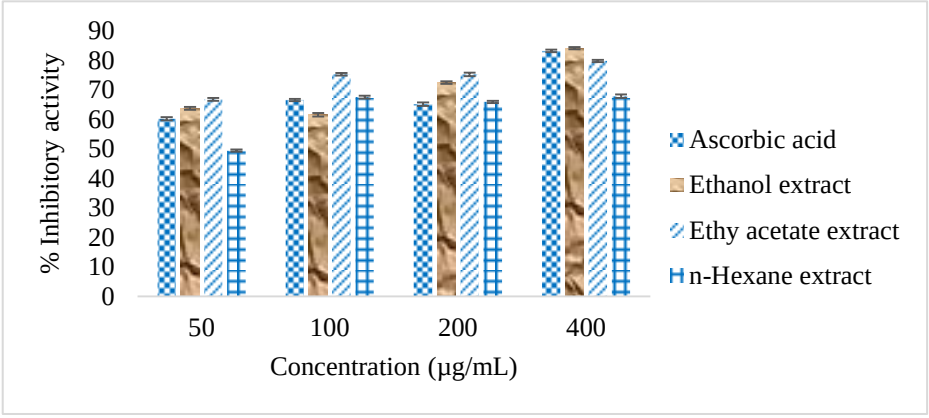

Figure 3: Percentage inhibitory activities of ethanol, ethyl acetate and n-hexane extracts of *M. indica* leaves on lipid peroxidation. Values are presented as mean ± standard deviation of triplicates. Values with different superscripts at the same concentration are significantly different at $p < 0.05$.

Discussion

DPPH is a suitable free radical compound widely used for testing the free-radical scavenging activities of different types of samples. The DPPH radical scavenging test largely depends on the ability of a compound to donate hydrogen atom; thus stabilizing the free radicals which in turn prevent oxidation of biomolecules (Kusuma et al. 2014). Although the DPPH radical does not conform with any biological compounds (and thus has a relatively minute relevance in living organisms), nevertheless, DPPH assay is generally considered as an indicator of the ability of plant extracts to quench free radicals, as well as their hydrogen atom or electron donation ability, in the absence of any enzymatic action (Mileva et al. 2014). The most advantageous reason for the use of DPPH in evaluating *in vitro* antioxidant activities of drugs and/or plant extracts is due to its higher stability than hydroxyl and superoxide radicals (Li et al. 2009). Hence, the antioxidant activities exhibited by the extracts via DPPH scavenging capacities may be said to be predominantly due to their hydrogen atom or electron donation ability. The hydrogen-donating ability, on the other hand, may be traceable to the presence of phenolic compounds in the extracts as these secondary metabolites have been found to possess antioxidant activities (Gruz et al. 2011). Therefore, it is rational to deduce that the higher activity of the ethanol extract than the ethyl acetate and n-hexane extracts may be as a result of higher concentrations of phenolics in the ethanol extract. This may be also true for the higher activity of the ethyl acetate extract than n-hexane extract.

The electron donating ability of antioxidants in plant extracts is usually reflected by the ability of such antioxidants to

reduce iron (Fe) in the oxidation state of Fe^{3+} to Fe^{2+}. Thus, the higher the activity of the antioxidants denotes the higher electron donating ability (reducing ability) (Amari et al. 2014). Therefore, the significant activities of the extracts imply that they were able to reduce Fe^{3+} to Fe^{2+}, revealing their electron-donating ability, which in turn implies the possibility of using the extracts in preventing oxidation of biomolecules in cells. The results obtained for the ethanol extract varied from those reported by Udem et al (2018), in the sense that the percentage inhibition obtained in this study was higher. The same reasons mentioned in the DPPH radical scavenging assay may also be responsible for the differences in this assay. Nevertheless, in both studies, ethanol extract of *M. indica* presented significant ferric reducing power.

Lipid peroxidation has been described as an oxidative breakdown of lipids, a process in which free radicals abstract electrons from cell membrane lipids (this mostly affects polyunsaturated fatty acids due to the presence of double bonds). The process of lipid peroxidation has been proposed to occur via a free radical chain reaction, which has been implicated in the damage of cell biomembranes (Halliwell 1989). The damage caused has been confirmed to predispose victims to numerous diseases, such diseases as cardiovascular diseases, cancer and diabetes (Usuki 1981). Therefore, the ability of the extracts to significantly inhibit lipid peroxidation of the egg homogenate means that they were able to quench the actions of the free radicals by preventing the abstraction of the electrons from cell membrane lipids by the free radicals and thus may be used in shielding humans from aforementioned diseases and other oxidative stress related diseases.

The significant inhibitory activity exhibited by the ethanol extract on DPPH radical, and its ferric reducing power correlates with the report of Udem et al (2018), however, the values obtained in this study were higher. The difference may be as a result of different concentration ranges used, method of extraction or different sites of sample collection. As for the inhibitory activity of the ethanol extract on lipid peroxidation, the values reported by Udem et al (2018) were higher than the ones reported in this present study. Nevertheless, in both studies, the ethanol extract of *M. indica* presented significant inhibitory activities. The reasons for this difference may also be the same for the higher values recorded for DPPH radical assay and FRAP assay in this study.

Conclusion

The results obtained in this study suggest that the ethanol, ethyl acetate and n-hexane extracts of *M. indica* possess antioxidant activities and may therefore be used in the treatments and/or management of oxidative stress related diseases.

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