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ANTIOXIDATIVE ACTIVITY OF THE ALMOND LEAVES (*TERMINALIA CATAPPA*)

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ABSTRACT: *The antioxidative activity of almond leaf was evaluated by using 1,1-diphenyl-2-picrylhydrazyl (DPPH),total phenolic content and reducing power assays. The 80% methanolic leaf extracts had the highest percentage inhibition (73.42%) of free radical followed by water and then 95% ethanol extract. The IC_{50} values of 80% methanol (10.00mg/ml) was significantly lower than that of the standard, ascorbic acid (12.45mg/ml) and this reflected the ease with which they mop/scavenge free radicals. The three different solvent extracts from almond leaf had demonstrated its antioxidative action by scavenging (inhibiting) DPPH radical, with appreciable amount of phenols and its concomitant reducing activity. The DPPH and TPC had the same order: 80%methanol > water > 95% ethanol whereas the reducing power had 80% methanol > 95% > water. Almond leaf could also be used as natural and phytotherapy in the management of oxidative stress-related diseases.*

KEYWORDS: Antioxidant, Free Radical, Almond Leaf

INTRODUCTION

Almond (*Terminalia catappa*) is one of the species of *catappa* belonging to the family of *Combretaceae*. Nutritionally and medicinally, almond is a valuable food commodity. In addition to low density lipoprotein (LDL) and Cholesterol lowering effect, the consumption of almond is also associated with the reduced risk of heart diseases. Such health functions related to almond consumption can be attributed to the antioxidant activity of vitamin E and monounsaturated fats as well as the presence of phenolics such as catechin, protocatechuic acid, prenylated benzoic acid, 2-prenylated benzoic acid, and 2-prenyl-4-O-ß-D-glucopyranosyl-oxy-4-hydroxybenzoic acid in the stem, root and leaf part of the almond plant (Subashinee *et al*., 2002). Almond plant (*T. catappa)* is widely grown in tropical regions of the world as ornamental tree, grown for the deep shade that its leaves provide .The fruit is edible, tasting slightly acidic. The wood is highly water resistant and is used in making canoes. The leaves, barks and roots are used in traditional medicine for various purposes.

The use of synthetic antioxidants such as butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA) , gallic acid esters and tertiary butylated hydroquinone (TBHQ) is becoming limited in the food industry due to their perceived carcinogenic potential (Jeong *et al.*, 2004). Contrary, plant-derived natural antioxidants, because of the anticarcinogenic attributes and other related medicinal benefits, are gaining much appreciation (Iqbal *et al.*, 2005; Sultanna *et al.*, 2009). Plants are recognized as one of the most potential sources of natural antioxidants (Shahidi, 1997). Various studies and books revealed that the antioxidant potential of plants are due to the occurrence of different valuable bioactives, especially the phenolic compounds (Shumalia *et al*., 2012; Shahidi, 1997). The role of dietary antioxidants including vitamin C, carotenoid, tocopherols and polyphenols in improving the health is

well accepted, supporting the fact that diets rich in fruits and vegetables are associated with the reduced risk of chronic diseases (Lim *et al.,* 2002 ; Onigbinde , 2005). Regular use of vegetables, nuts and sprouts in the diet can be associated to reduce the risk of certain diseases including cancer, diabetes, atherosclerosis, aging-related disorders and inflammation. (Pinelo *et al.,*2004). Such health promoting properties of leaves might be linked to the presence of bioactive compounds (antioxidants) such as flavonoids and other phenolics (Subashinee *et al*.,2002). Dietary antioxidants including polyphenolic compounds, Vitamin C, E and carotenoids are believed to be the effective nutrients in the prevention of these oxidative stress related diseases. Antioxidants have thus become a topic of increasing interest.

Therefore, it is very appealing to the researchers to have a convenient method for the quick quantification of antioxidant effectiveness in preventing diseases (AOAC, 1996).Different solvent systems have been used for the extraction of the antioxidant components from the almond leaf. The antioxidant activity of the extracted almond (*T. catappa*) plant materials are strongly affected by the nature of extraction solvents (Anwar *et al*., 2010; Sultanna *et al*., 2009).As the almond (*T. catappa)* is a potential source of bioactives, it would be interesting to evaluate the efficacy of the different extraction solvents towards recovery of potent antioxidants from the almond leaves.

There is no work yet that had compared the effectiveness of these solvents at the given concentrations**.** Although, this is part of my MSc thesis.

Figure 1: Amond plant (Terminalia catappa)

LITERATURE/THEORETICAL UNDERPRINNING

Mechanisms Of Free Radical Reactions

The nature of free radical reactions, like other forms of chemical reactions occur in stages which are: initiation, propagation and termination. The initiation and propagation stages involve:

Free radical reacts with another free radical: if two radicals meet, they can join their unpaired electrons and make a covalent bond (a shared pair of electrons), (Onigbinde, 2005)

Thus, superoxide radical reacts with nitric oxide radical to form peroxynitrite radical

$$OO\bullet + NO\bullet \longrightarrow ONOO\bullet$$

Superoxide Radical — Nitric Oxide Radical — Peroxynitrite radical

At physiological pH, peroxynitrite can damage proteins directly and will decompose into products which include nitrogen dioxide gas and the noxious hydroxyl radical, OH•. Hence, at least some of the toxicity of excess nitric oxide radical, NO• may involve its interaction with superoxide radical. Excess superoxide radical can in turn react with iron and copper ions, thereby causing more damage. Peroxynitrite radical is a versatile oxidant; it oxidizes unsaturated fatty acids, ascorbic acid, tocopherol, uric acid and amino acids among other substances. It easily decomposes with the formation of radicals, which can start lipid peroxidation (Brannan *et al.*, 2001).

Radical reacting with Non-radical

Most molecules found in the human body are not radicals. Hence, any reactive free radical generated is most likely to react with a non-radical. When a free radical reacts with a non-radical, a free radical chain reaction results and a new radical is formed (Onigbinde , 2005).

$$RO_2\bullet + AH \longrightarrow ROOH + A\bullet$$

radical — non-radical — non-radical — radical

Attack of reactive radicals upon membranes or lipid proteins starts the free radical chain reaction called lipid peroxidation (Belitz *et al.,* 2009). There is growing evidence that lipid peroxidation takes place in human blood vessel walls and contributes to the development of atherosclerosis, raising the risk of stroke and myocardial infarction (Choe *et al*., 2006). If hydroxyl radicals (OH•) generated are close to DNA, they can attack the purine and pyrimidines and it can cause mutation. The purine-guanine is converted into 8-hydroxyl guanine and other products which can cause errors during DNA replication (Allen *et al*., 1996).

$$RO_2^{\bullet} + AH \longrightarrow ROOH + A^{\bullet} \quad \text{(i)}$$

$$RO\bullet + AH \longrightarrow ROH + A^{\bullet} \quad \text{(ii)}$$

$$RO_2^{\bullet} + A\bullet \longrightarrow ROOA \quad \text{(iii)}$$

$$RO^{\bullet} + A\bullet \longrightarrow ROA \quad \text{(iv)}$$

Activity of an antioxidant as a radical scavenger**.** Where AH = Antioxidant

ANTIOXIDANT DEFENCE IN HUMAN BODY

Free radicals are constantly made in large amounts in the human body, antioxidant defense have been evolved to protect the body tissues. Superoxide dismutase (SOD) enzyme removes

Superoxide radical (O_2•) by converting it to hydrogen peroxide (H_2O_2) and oxygen (Choe, *et al*., 2006).

$$2O_2\bullet + 2H \xrightarrow[\text{Enzyme}]{\text{SOD}} H_2O_2 + O_2$$

Hydrogen peroxide is mostly removed by catalase and glutathione peroxidase enzymes. Some metallic ions such as iron and copper ions are powerful promoters of free radical damage by accelerating lipid peroxidation. The human body has a complex system of metal ion transport and storage proteins to ensure that these essential metals are rarely allowed to be in the Free State (Onigbinde, 2005). Also in the body are molecules such as glutathione, α- tocopherol and ascorbate that remove free radicals by reacting directly with them in a non-catalytic manner, some of these free radical scavengers come from the diet (Belitz *et al.*, 2009). Despite all these antioxidants, some free radicals still escape to cause damage in the human body (Onigbinde, 2005). Free radicals have to be degraded and the antioxidant nutrients play vital roles in counter-reacting the effect of free radical activity. The end product of lipid peroxidation are measurable in human body fluids, in atherosclerotic lesion, and in the age pigments that accumulate in old tissue and thus , initiation of heavy metal-catalyzed lipid autoxidation can be prevented (Dhalwal *et al*., 2005).

MATERIALS AND METHODS

Collection Of Samples

Samples of the leaves, stems and roots of the tropical almond plant (*Terminalia catappa*) were collected from the Botany Department of Ambrose Alli University (AAU), Ekpoma,Edo state,after being identified and confirmed by a plant taxonomist.

Chemicals And Reagents

All the reagents used in the present experiment were of analytical grade from Merck, Fluka, Aldrich or sigma, unless otherwise specified. 2,2- diphenyl-1-picrylhydrazyl free radical (DPPH), Gallic acid, Folin-ciocalteu reagent (FCR), ferric chloride hexahydrate ($FeCl_3.6H_2O$), anhydrous sodium carbonate (Na_2CO_3) were purchased from sigma chemicals Co (St. Louis, M.O, USA); potassium dihydrogen phosphate, dipotassium hydrogen phosphate, L-Ascorbic acid, Ethanol, methanol were purchased from Merck (Darmstadt, Germany) Food grade synthetic antioxidant-Butylated hydroxyl toluene (BHT), (+) - catechin hydrate, Hydrochloric acid were purchased from Fluka chemicals (Buch,Switzercland), Vanillin, potassium ferricyanide, Trichloroacetic acid (TCA), ammonium thiocyanate were purchased from Aldrich chemical company (Steinheim, Germany). All other reagents were of the highest quality grade available.

Experimental procedures

Ten grammes of the fresh samples of almond leaves were weighed using analytical chemical balance and ground to fine powder using Laboratory mortal and pestle. Three solvent systems namely: 80% methanol (methanol: water, 80:20 v/v), 95% Ethanol (Ethanol: water, 95:5 v/v) and water (100% water) were employed for the extraction purpose. The ground material

(10g) was extracted with 50ml of each of the extracting solvents (80% methanol), (95% ethanol) and 100% water at room temperature for 48hrs. The residues were separated from the extracts by filtering through filter paper (Whatman No.1); the residues were further extracted with fresh solvent. The extracts recovered from both the extractions were pooled from the solvents under vacuum at 45^0C, using a rotary evaporator. The crude concentrated extracts were stored in a refrigerator (4^0C), until used for further analyses (Sultanna *et al.*, 2009).

DPPH radical scavenging assay

1, 1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of the almond extracts were assessed in triplicate using the protocol by lqbal et al. (2005) and Bac (2007). A 0.12mM DPPH solution (Sigma, USA) was prepared in 80% methanol and then 1ml of this solution was added to 500 µl of the different concentrations of vitamin C (L-ascorbic acid) as a standard. The mixture was vortexed for 15 seconds and incubated in water bath at 37^0C for 30 mins. The absorbance was read at 517 nm. The above steps were repeated by replacing vitamin C with the different concentrations of the leaf extracts. The initial sample extract (in ml) was diluted with the specific volume of the methanol to prepare the required concentration of the leaf, stem and root extracts. The IC_{50} values were calculated from the graph of the concentrations of the vitamin C against the percentage inhibition of the sample extracts. The IC_{50} which represents concentrations of the sample extracts required to provide 50% inhibition of the DPPH free radicals. The percentage inhibition of DPPH radical scavenging activity was calculated using the equation:

$$\%I = \frac{(A_{control} - A_{sample})}{A_{control}} \times \frac{100}{1}$$

In this equation; $A_{control}$ denotes the absorbance of control while A_{sample} is the absorbance of the test reaction mixture.

Reducing Power Assay

The reducing power of the almond leaf, extracts were assessed in triplicate according to the procedure described by Yen *et al.* (2000).Exactly 0.2ml of the extract of different concentrations was mixed with 200 µl of 1% potassium ferricyanide. The mixture was incubated in the water bath for 20 min at 50^0C. Trichloroacetic acid (250 µl) was added to the mixture and was centrifuged at 1000 rpm for 10 min at room temperature. The supernatant (500 µl was added with 500 µl of distilled water and 100 µl of 0.1% ferric chloride. the mixture was incubated in the oven at 37^0C for 10mins. Absorbance was recorded at 700nm using a Shimadzu UV-210A double - beam Spectrophotometer. Ethanolic solution of known Fe (II) concentration in the range of 50-500µM ($FeSO_4$) were used as calibration curve. The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1mM $FeSO_4$ and butylated hydroxytoluene (BHT) was used as a positive control(Yen *et al.*,2000).

Determination of Total Phenolic Content (TPC)

Total phenolic content of the almond leaf was determined in triplicate using Folin-Ciocalteu assay (Sultanna *et al.*, 2009).In this test, different aliquots (0.1,0.3,0.5 and 0.7ml) of the crude extracts were made up to 1.0 ml with water before 0.4ml of Folin –Ciocalteu reagent

was added. The mixture was kept at room temperature for 10min and then 0.3ml of 20% sodium carbonate (w/v) was added. The mixture was further incubated for two hours at room temperature. Subsequently, absorbance was read at 750nm using a Shimadzu UV-210A double beam spectrophotometer. A standard calibration curve was drawn using gallic acid of different concentrations (0.1, 0.3, 0.5, and 0.7ml/ml) .The steps above were repeated replacing gallic acid concentrations with the leaf extract concentrations. With absorbance of sample extracts, results were extrapolated from the standard curve. The total phenolic content was expressed as milligram of gallic acid equivalent (GAE) per gram of fresh weight.

Statistical Analysis

The data obtained were analyzed by one – way analysis of variance (ANOVA) using Turkey – Kramer multiple comparisons test. P values less than 0.05 were considered to be statistically significant.

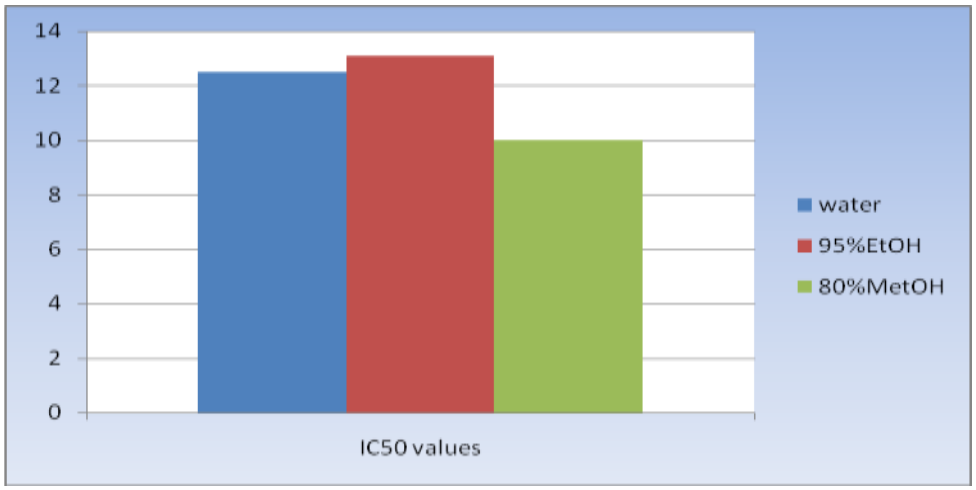
RESULTS/FINDINGS

Table 4.1: Percentage inhibition (%) of DPPH free radical by the Almond Leaf extracts

Extract Conc(ml/ml)	Water	95%Ethanol	80% Methanol
0.1	35.17^{a}	27.08^{b}	35.30^{a}
0.2	40.20^{b}	30.00^{c}	53.05^{a}
0.3	43.58^{b}	35.12^{c}	65.78^{a}
0.4	50.36^{b}	40.67^{c}	70.00^{a}
0.5	54.21^{b}	48.10^{c}	73.42^{a}

Means with the same superscript in a row are not significantly different.

DPPH represents 1,1-diphenyl-2-2picrylhydrazyl.



IC_{50} value for the standard, ascorbic acid is 12.45 mg/ml. EtOH and MetOH represents ethanol and methanol respectively.

Figure 2: The IC_{50} value (in mg/ml) for the different Almond leaf extracts.

Table 4.2: Reducing power of Almond leaf extracts in milligram of butylated hydroxyltoluene (BHT) equivalent per gram of fresh weight

Extract Conc (ml/ml)	Water	95% Ethanol	80%Methanol
0.1	6.92^{c}	8.00^{b}	11.02^{a}
0.3	12.67^{c}	14.92^{b}	15.58^{a}
0.5	15.75^{b}	17.00^{a}	17.33^{a}
0.7	20.00^{b}	21.20^{a}	21.69^{a}

Means with the same superscript in a row are not significantly different

Table 4.3: Total phenolic content of the almond leaf extracts in mg of GAE/g of fresh weight

Extract Conc(ml/ml)	Water	95% Ethanol	80%Methanol
0.1	43.70^{b}	42.50^{c}	45.00^{a}
0.3	51.70^{b}	49.20^{c}	56.70^{a}
0.5	67.60^{bc}	66.70^{bc}	70.00^{a}
0.7	112.20^{b}	106.70^{c}	159.00^{a}

Means with the same superscript in a row is not significantly different; GAE represents gallic acid equivalent.

DISCUSSION

DPPH scavenging activity

The free radical scavenging activities of the 80% methanolic leaf extracts was significantly higher than that of water and ethanol. There was positively correlation with its reducing potential, displayed by lower IC_{50} values which reflected the ease with which they mop/scavenge free radicals. The IC_{50} values were extrapolated from the linear regression plot of percentage inhibition against the concentration of vitamin C. The IC_{50} value of the different extracts is shown in figure 2.The methanolic leaf extracts had the strongest DPPH scavenging ability followed by water extracts and then ethanolic extracts: 80% methanol > water > 95% ethanol. The methanolic leaf extracts showed the strongest inhibition (73.42%) of the DPPH free radical at 0.5ml/ml. The methanolic leaf extract had significantly lower IC_{50} value(10.00mg/ml) than that of the standard, ascorbic acid(12.45mg/ml),whereas water and ethanolic extracts have significantly higher IC_{50} values than the standard, ascorbic acid. The lower the IC_{50} value, the higher the scavenging activity. The IC_{50} represents the concentration of the sample extract required to scavenge 50% DPPH radical. The scavenging activity of the methanolic extracts was significantly higher ($P< 0.05$) when compared with water and ethanolic extracts. It follows that the scavenging activity of water extracts were significantly higher than that of ethanolic extracts (table 4.1).This was in contrast with the previously reported data on the free radical of anthraquinones in *Russula delica* by Yen *et al.* (2000), and that aqueous ethanol extracts is preferable to water extracts in scavenging activity.

The result of this study agrees with the earlier publication reported for the free radical scavenging activity of *Hydroxyanmus squareous* by Ebrahimzadeh *et al.*(2009).The observed higher scavenging ability of the methanolic extracts proved the efficacy of this solvent and

supports the previous studies in the literature which showed that antioxidant compounds from plant sources were best extracted by seventy to eighty percent (70 - 80%) alcohols .

Reducing power

The reducing power of the three different almond leaf extracts is an important indicator of their potential antioxidant activities. The result in table 4.2 shows the reducing potentials of the different solvent extracts from almond leaf. The reducing power of the extracts increased with increase in the extracts concentrations. In comparison, at 0.5 and 0.7ml/ml there were no significant difference ($P<0.05$) between methanolic and ethanolic leaf extracts in terms of their reducing potentials. There was a slight variation in the pattern of reducing ability with that of scavenging activities. This variation may be due to bioavailability of the antioxidant substances, interactions between the plant cells and the solvents, and other environmental factors. The result in table 4.2 demonstrated the order: 80% methanol > 95%ethanol > water. Almond leaf is a good reductone and can exert its antioxidant activity by donating a hydrogen atom and thereby breaking the free radical chains.

Total Phenolic Content

The total phenolic contents of the different solvent extracts from almond leaf were compared as shown in table 4.3. The amount of total phenols from different almond leaf extracts differ significantly. The 80% methanolic extracts contained higher phenols than water and 95% ethanol. All the total phenolic contents of the three different solvent extracts from almond leaf increased as the concentration of the extracts increases. The methanolic extracts have recorded significantly higher ($P<0.05$) total phenolic contents than the other counter parts. Thus, 80% methanol > water > 95% ethanol. This result supported several reports by Shumalia *et al*. (2012) that the solubility of phenolic compounds depends on the chemical nature of the plant tissues and the polarity of the solvent system. 80% methanol extracts had better antioxidant activities than 95% ethanol extracts. This may be explained by the inability of 95% ethanol to extract phenolic compounds which are more water soluble (hydrophilic).The presence of water in the extraction eases the release of hydrophilic antioxidants(Siddhuraju *et al*.,2003; Sultana *et al*.,2009). The three different solvent extracts from almond leaf had demonstrated its antioxidative action by scavenging (inhibiting) DPPH radical, with appreciable amount of phenols and its concomitant reducing activity. The study observed that 80% methanolic extracts of the three almond leaf tested were better in scavenging activity, contains higher reducing power and total phenolic content than 95% ethanol and water. This finding agrees with the report on the antioxidant properties of *Prunus dulcis* by Shumalia *et al*. (2012).

IMPLICATION TO RESEARCH AND PRACTICE

The almond plant is readily available and so there is need to further explore its potential as possible source of bioactive compounds with antioxidant properties. Almond leaf could also be used as natural and phytotherapy in the management of oxidative stress-related diseases.

CONCLUSION

The study has demonstrated that 80% methanolic leaf extracts from the almond plant possess higher antioxidant activity than 95% ethanol and water extracts. The results also showed that

the phenols of almond leaf may be partly responsible for the observed scavenging activity. The results of this study support the recent epidemiological studies that the consumption of certain plant materials such as almond seeds (nuts), leaves, fruits, stems and roots may reduce the risk of chronic diseases related to oxidative stress, on the account of their antioxidant activity and promote general health benefits.

Future Research

Further characterization of the bioactive compounds is suggested as theme for further studies

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