In Vitro and In Vivo Antioxidant Activity of Aqueous and Ethanol Leaf Extracts of Murraya Koenigii

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ABSTRACT
Reactive oxygen species generated from certain cellular processes cause tissue and cell damage and may alter the structure and functioning of proteins and nucleic acids. Endogenous antioxidant defence mechanisms involving certain enzymes as well as exogenous antioxidant from plants are capable of preventing such complications. This study investigated the in vitro and in vivo antioxidant activity of Murraya koenigii (curry leaf) extracts. Water and ethanol extracts of the leaf were obtained by soxhlet extraction. In vitro antioxidant activity was by the ferric reducing power and hydrogen peroxide scavenging assays and the total flavonoid and flavonol levels determined. The in vivo activity was assessed using albino wistar rats which were treated with 100mg/kg of aqueous and ethanol leaf extracts and ascorbic acid as positive control for six consecutive days after which a single dose (2.5mL/kg body) of CCl₄ was administered except for the normal control group 24hrs later. The animals were sacrificed and the liver homogenate was used to assess the catalase activity and lipidic peroxidation. Aqueous and ethanol extracts showed ferric reducing activity and were concentration dependent. The hydrogen peroxide scavenging potential was very high at various concentrations for both extracts (between 81.2 to 99.9%) comparable to ascorbic acid. Molondialdehyde (MDA) level (lipidic peroxidation) was significantly lower (p<0.05) in group 2 animals which received aqueous extract compared to group 1 (normal control) and group 4 (negative control). There was a significant difference (p<0.05) in the catalase activity of group 2 animals which received aqueous extract compared to the normal control. Acute toxicity was observed in group 5 animals administered ethanol extract. Flavonoid and flavonol were greater in the ethanol extract (625.33 and 835.04 g/g of rutin) than in the aqueous extract (449.33 and 100.33 g/g of rutin) respectively. These results suggest that Murraya koenigii (curry leaf) aqueous and ethanol extracts possess antioxidant activity due to high amounts of flavonoid and flavonol. This property may partly be responsible for the medicinal application of the plant.

Keywords: oxidative stress, Murraya koenigii extracts, catalase, lipid peroxidation, flavonoid, flavonol, CCl₄

INTRODUCTION
Oxidative stress, an unhealthy condition is widely known to significantly contribute to several human diseases such as inflammatory diseases, ischemic diseases, acquired immunodeficiency syndrome, gastric ulcers, hypertension, neurological disorders, diabetes, multiple sclerosis etc.¹,². Oxidative stress is also known to be one of the major sources of carcinogenesis, the top-ranked cause of human mortality worldwide³. Defined by an overwhelmed level of pro-oxidants out numbering the antioxidant capacity in cells, oxidative stress is as a result of overproduction of free radicals or decrease in antioxidant defence systems⁴,⁵. Free radicals are usually molecular species capable of independent existence such as hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical etc.⁶,⁷. They contain an unpaired electron in an atomic orbital which are unstable and highly reactive species, capable of attacking the nucleus and membranes of cells and damaging biologically relevant molecules such as DNA, proteins, carbohydrates and lipids⁸. They can cause substantial damage to these macromolecules through the generation of adducts, destruction of unsaturated C-C bonds, and oxidation of disulfides⁹. Free radicals are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals¹⁰,¹¹. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non enzymatic reactions. On the other hands, antioxidants which are molecules stable enough to donate an electron to a rampaging free radical, can remove or neutralize it, thus reducing its capacity to damage¹². Hence, free radicals which are responsible for oxidative stress can be removed or regulated by antioxidants. These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged¹³. Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non enzymatic

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The dried leaves were ground into powdery form using an electric blender. 60g each of plant material was extracted using 500ml of ethanol and 1L of distilled water in a soxhlet extractor. The extracted solution containing the plant extract was evaporated at 45°C for 48 hours to obtain a yield of 7.5g and 22.5g respectively. **In vitro antioxidant activity**

**Determination of reducing power**

The reducing power of the extract was evaluated according to the method of Yen and Chen\textsuperscript{26}. A volume of 1.0 ml of the aqueous and ethanol extracts and ascorbic acid (0.125 - 1.0 mg/ml) each were mixed individually to a mixture containing 2.5ml of 0.2 M phosphate buffer (pH 7.0) and 2.5 ml of potassium ferricyanide (K₃Fe(CN)₆) (1% w/v). The resulting mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5ml of trichloroacetic acid (10% w/v), which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with 2.5 ml of distilled water and 0.5 ml of ferrous chloride (0.1% w/v). The absorbance was measured at 700nm against a blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

**Hydrogen peroxide scavenging activity**

Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method of Ruch, Cheng and Klaunig\textsuperscript{27}. A volume of 4ml of aqueous and ethanol extracts and ascorbic acid at various concentration (0.125 - 1.0 mg/ml) each was mixed with 0.6 ml of 4 mM H₂O₂ solution prepared in phosphate buffer (0.1 M pH 7.0) and incubated for 10 min. The absorbance (Abs) of the solution was taken at 230 nm against blank solution containing the plant extract without H₂O₂. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation:

\[
\text{H}_2\text{O}_2 \text{ radical scavenging activity} = \{\text{Abs control} - \text{Abs sample}\}/\text{Abs control} \times 100
\]

Where; Abs control is the absorbance of H₂O₂ radical + solvent; Abs sample is the absorbance of H₂O₂ radical + sample extract or standard.

**In vivo antioxidant activity**

**Animals and handling**

Adult wistar albino rats weighing between 120g and 200g were obtained from the animal house of Faculty of Biological Sciences, University of Nigeria, Nsukka and transported to the animal house of Godfrey Okoye University Enugu, Enugu State of Nigeria. The animals

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**MATERIALS AND METHODS**

**Collection of plant leaves**

Fresh curry leaves identified botanically by as *Murraya koenigii* were purchased from Abakpa market, Enugu East local government area of Enugu State Nigeria. The leaves of *Murraya koenigii* were air dried at room temperature in the chemistry laboratory of Godfrey Okoye University for 7 days.

**Preparation of plant extract**

Antioxidants exist in the intracellular and extracellular environment to detoxify reactive oxygen species (ROS)\textsuperscript{14}. The enzymatic detoxification processes is initiated firstly by superoxide dismutase (SOD) which catalyses the breakdown of superoxide released during oxidative phosphorylation to hydrogen peroxide and oxygen\textsuperscript{15,16}. Catalase and glutathione peroxidase remove hydrogen peroxide by decomposing it to water and oxygen\textsuperscript{17,19}. Some of the non enzymatic antioxidants which are produced during normal metabolism in the body include glutathione, ubiquinol, and uric acid\textsuperscript{20}. Other lighter antioxidants cannot be manufactured by the body so they are supplied by the dietary sources\textsuperscript{21} or medicinal plants. These include vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene, polyphenols, flavonoids, flavanol etc. *Murraya koenigii* belonging to the family Rutaceae is native to India and is usually found in tropical and subtropical regions. It is cultivated in other countries such as China, Australia, Nigeria and Ceylon\textsuperscript{22}. Locally known in Nigeria as curry leaf, is a natural flavouring agent with a number of important health benefits, which makes our food both healthy and tasty along with pleasing aroma. They are known for various medicinal properties to control diarrhea, gastrointestinal problems such as indigestion, excessive acid secretion, peptic ulcers, dysentery, diabetes and an unhealthy cholesterol balance\textsuperscript{23}. They are also believed to have cancer fighting properties and are known to help protect the liver\textsuperscript{24}. *Murraya koenigii* is rich in nutrients such as carbohydrates, energy, fiber, calcium, phosphorous, iron, magnesium, copper and minerals. It also contains various vitamins like nicotinic acid and vitamin C, vitamin A, vitamin B, vitamin E, plant sterols, amino acids, glycosides and flavonoids\textsuperscript{25}. The various medicinal applications of *Murraya koenigii* and presence of vitamins and flavonoids which are known to have antioxidant properties prompted this study.

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**Figure 1: Experimental design chart**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1-6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>Distilled water</td>
<td>Distilled water</td>
<td>Animals sacrificed and liver collected for catalase activity and lipidic peroxidation determination</td>
</tr>
<tr>
<td>Experimental Group</td>
<td>Aqueous extract</td>
<td>CCl₄</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 3</td>
<td>Group 4</td>
</tr>
<tr>
<td>Positive control</td>
<td>Ascorbic acid</td>
<td>Distilled water</td>
<td>Ethanol extract</td>
</tr>
</tbody>
</table>

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**Table 1: Preparation of the sample extract**

The sample extract of *Murraya koenigii* was prepared in a yield of 7.5g and 22.5g respectively.
were acclimatised for a duration of 14 days under standard environmental conditions with a 12 hour light/dark phase and were allowed access to food (top feeds, growers mash) and water ad libitum twice daily.  

Animal grouping and treatment

A total of 25 rats were randomized into five groups consisting of five animals each and orally administered 0.5 ml of treatment daily for seven days. Group 1 and 4; the normal and negative controls respectively were given distilled water, group 3; the positive control received
ascorbic acid (vitamin C) while group 2 and 5; the experimental groups were given 100mg/kg body weight aqueous and ethanol extracts respectively.

Inducement of liver damage
On the seventh day about 6 hours after treatment administration, groups 2 to 5 were treated intraperitoneally with 0.5ml of a single dose of 2.5mL/kg body weight of Carbon tetrachloride (CCl₄) and olive oil (1:1). Group 1 was given distilled water/olive oil (1:1). Collection of Samples from Animals
Following an overnight fasting for 24 hours, the animals were sacrificed by cardiac puncture under mild anaesthesia using chloroform and the liver from each animal was excised and rinsed with normal saline. Inducement of liver damage
The activity of catalase was assayed following the method described by Pari and Latha. The percentage inhibition was calculated using the equation:

\[
\frac{\text{normal activity } - \text{ inhibited activity}}{\text{normal activity}} \times 100\%
\]

Where:
Normal activity = hydrogen peroxide + phosphate buffer; Inhibited activity = hydrogen peroxide+ phosphate buffer + liver homogenate

Estimation of lipid peroxidation
Lipid peroxidation in the liver was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modification method of Niehuis and Samuelsson. In brief, 0.1 ml of liver homogenate (10% w/v) was treated with 2 ml of (1:1:1 ratio) TBA- TCA-HCl reagent (thiobarbituric acid 0.37%, 15% trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min and cooled. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the absorbance of clear supernatant at 535nm against reference blank. Concentration of MDA was calculated using the equation:

\[
C = \frac{E \times L}{A}
\]

Where: A is the absorbance of the sample, E is the extinction coefficient (1.56 x 10³ M⁻¹ cm⁻¹) and L is the length of the light path (1cm)

Estimation of polyphenol compounds
Total flavonoids
The method of Ordon Ez et al. was used to estimate total flavonoids contents of the extract solution based on the formation of a complex flavonoids-aluminium. A volume of 0.5ml of 2% AlCl₃ ethanol solution was added to 0.5ml of extract solution. After one hour of incubation at room temperature, the absorbance was measured at 420nm using UV-VIS spectrophotometer. All determinations were done in triplicate and values were calculated from the calibration curve of rutin (Figure 2) using the equation:

\[
Y = 0.001X + 0.030, R^2 = 0.910
X = Y – 0.030
\]

Where: Y = absorbance, X = concentration of rutin (mg/g)

Total flavonol
Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran. The reacting mixture consisted of 2.0 ml of the sample, 2.0 ml of AlCl₃ prepared in ethanol and 3.0 ml of (50 g/l) sodium acetate solution. The absorption at 440 nm was read after 2.5 h at 20°C. Total flavonol content was calculated as rutin (mg/g) equivalent from the calibration curve of rutin (Figure 3) using the equation:

\[
Y = 0.004X + 0.129, R^2 = 0.973
X = Y – 0.129
\]

Where: Y = absorbance, X = concentration of rutin (mg/g)

Statistical Analysis
Table 1: Catalase activity and lipidic peroxidation in various treated animal groups

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (% Inhibition)</td>
<td>27.83±4.35</td>
<td>26.68±2.20</td>
<td>31.93±2.59</td>
<td>30.29±3.13</td>
<td>0.580</td>
</tr>
<tr>
<td>Lipidic peroxidation (µM)</td>
<td>3.67±0.33</td>
<td>3.25±0.48</td>
<td>4.40±0.51</td>
<td>4.50±0.65</td>
<td>0.323</td>
</tr>
</tbody>
</table>

Table 2: Total flavonoid and flavonol content in plant extract

<table>
<thead>
<tr>
<th></th>
<th>Aqueous extract</th>
<th>Ethanol extract</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>476</td>
<td>686</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>464</td>
<td>608</td>
<td></td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>582</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>449.33±20.96</td>
<td>625.33±31.25</td>
<td></td>
</tr>
<tr>
<td>Flavonol</td>
<td>91</td>
<td>771.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101.5</td>
<td>868</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>108.5</td>
<td>865.38</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>100.33±8.81</td>
<td>835.04±54.88</td>
<td></td>
</tr>
</tbody>
</table>

The data obtained were analyzed using statistical package for social sciences (SPSS) version 16.0 and the results expressed as mean ± standard error. Significant differences were established by one way analysis of variance (ANOVA) and the acceptance level of significance was P≤0.05.

RESULTS

In vitro antioxidant activity
The extracts of Murraya koenigii (aqueous and ethanol extracts) showed ferric reducing power though not as high as that of ascorbic acid. For the various concentrations, the absorbance at 700nm for aqueous extract ranged from 0.218 to 0.681, the ethanol extract was from 0.413 to 0.596 and ascorbic acid from 0.637 to 1.363. The ferric reducing power for both extracts as well as ascorbic acid increased with increasing concentration, thus was dose dependent (Figure 4).

Legend: AE: Aqueous extract EE: Ethanol Extract AS: Ascorbic acid Both the aqueous and ethanol extracts of Murraya koenigii showed very high hydrogen peroxide scavenging activity comparable to that of ascorbic acid. The ethanol extract had a higher percentage inhibition of hydrogen peroxide than the aqueous extract for the various concentrations. The hydrogen peroxide scavenging activity was very high for both extracts at a very low concentration of 0.125mg/mL but the aqueous extract showed the lowest activity (54.9%) at a concentration of 1mg/mL (Figure 5).

In-vivo antioxidant activity
Following the administration of plant extracts to the various animal groups, acute toxicity was observed in all animals of group 5 administered ethanol extract and also in a few animals in group 4 (negative control) after CCl4 administration. Catalase activity was observed in all the animal groups and was highest (31.93±2.59%) in group 3 (positive control). The catalase free radical percentage inhibition in group 2 animals administered aqueous extract though low (26.68±2.20), was comparable to that of the normal control (27.83±4.35) and significantly different (p<0.05) from the negative control (30.29±3.13). The quantity of malondialdehyde formed was used to determine the degree of lipidic peroxidation. The malondialdehyde level (µM) was lowest (3.25±0.48) in group 2 animals administered aqueous extract comparable to the normal control (3.67±0.33) and significantly different (p<0.05) from the negative control (4.50±0.65). (Table 1). Legend: Group 1: Normal control, Group 2: Aqueous extract, Group 3: Positive control, Group 4: Negative control, S.E: Standard Error, a superscript indicates significant difference Total flavonoids and flavonols were present in different amounts in the aqueous and ethanol extracts. The concentration of flavonoid was significantly higher (p<0.05) in the ethanol extract (625.33±31.25) than in the aqueous extract (449.33±20.96). Also, the flavonol content was significantly higher (p<0.05) in the ethanol extract (835.04±54.88) than in the aqueous extract (100.33±8.81). (Table 2).

DISCUSSION
In view of increasing risk factors of human to various deadly diseases, there has been a global trend toward the use of natural substances present in medicinal and dietary plants as therapeutic antioxidants31. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers32. The Leaf of Murraya koenigii has been shown to have various medicinal properties33. Apart from its medicinal value, the leaf of this plant known as curry leaf in Nigeria is a local spice for various dishes and widely used as aavouring agent because of its pleasing aroma and taste. Because of its wide consumption, a potential antioxidant activity will be of great importance to the masses. Several in vitro techniques have been used to determine the antioxidant activity in order to allow rapid screening of substances with high antioxidant activity since substances that have low antioxidant activity in vitro, will probably show little activity in vivo. In this study, the ferric reducing power and hydrogen peroxide scavenging activity were assessed. Antioxidants have the ability to scavenge free radicals in vitro by reducing Fe3+ to Fe2+ through electron transfer ability34. This serves as an indicator of the antioxidant activity. The findings showed the aqueous and ethanol extracts of Murraya koenigii to possess in vitro ferric reducing activity though not as high as that of
ascorbic acid. The reducing power activity increased with increasing concentrations thus was concentration dependent. Hydrogen peroxide is a highly important reactive oxygen species because of its ability to penetrate biological membranes and damage them. Antioxidants can remove hydrogen peroxides by converting them to water. This study showed the ability of Muraya koenigii plant extracts to scavenge hydrogen peroxide in the reaction mixture. The percentage inhibition of both the aqueous and ethanol extracts was similar to that of ascorbic acid which is a known or reference antioxidant. This could be due to the presence of phenolic compounds that donate electron to H2O2 and thus neutralizing it to water. In vivo antioxidant assays are usually important to confirm the in vitro activity of certain antioxidant. To assess this in vivo antioxidant activity in animal models, a state of oxidative stress needs to be induced after the administration of plant extract to evaluate the ability of the plant extract to scavenge free radicals and prevent liver damage. Carbon tetrachloride (CCl4) is a substance that can cause oxidative stress by generating free radicals and thus one of the most commonly used hepatotoxins in experimental studies to cause liver damage. The hepatotoxic effects of this chemical is mostly based on membrane lipid peroxidation. Consequently, leads to the induction of trichloromethyl radical that results into severe cell damage. In this present study, the rats treated with single dose of CCl4 showed hepatic damage and lipidic peroxidation by an increase in the Malondialdehyde (MDA) level in group 4 animals (negative control) compared to group 1 (normal control). The MDA level was significantly lowest (p<0.05) in group 2 animals administered aqueous extract of Muraya koenigii which suggest that Muraya koenigii plant extract can prevent lipidic peroxidation and liver damage. Hence, has an antioxidant as well as a hepatic protective activity. Catalase is another antioxidant enzyme widely distributed in the animal tissues. It decomposes hydrogen peroxide to water and protects the tissues from highly reactive hydroxyl radicals. The reduction of activity of this enzyme may lead to deleterious effects as a result of hydrogen peroxide assimilation. In the present study, the percentage inhibition of catalase was significantly lower (p<0.05) compared to the negative control. Thus, the plant extract was shown to promote the production of catalase or enhance its activity. Acute toxicity was observed in the animals administered ethanol extract as they all died during the course of treatment. This may imply that ethanol may extract certain phytochemicals that are toxic to the animals. Also, a few casualties were observed in animals of the negative control group which were not treated before induction of liver damage with CCl4. Polyphenols are the major plant compounds with high level of antioxidant activity. This activity could be due to their ability to absorb, neutralize and to quench free radicals. Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation. Flavonoid and flavonol are plant-based compounds with powerful antioxidant properties found in many fruits and vegetables. In this present study, the leaf extracts of Muraya koenigii showed high level of flavonoids and flavonols. The ethanol extract showed a higher level of total flavonoids and flavonols compared to the aqueous extract which suggests that the type of solvent used is an important factor in the extraction of phytochemicals. In conclusion, Muraya koenigii aqueous and ethanol extracts possess both in vitro and in vivo antioxidant ability in scavenging free radicals which may be due to the presence of high levels of flavonoids and flavonols. This activity may partly be due to the presence of high levels of flavonoids and flavonols. The ability of this plant to prevent liver damage may be of interest for further studies to evaluate its potentials in the management of liver diseases.

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REFERENCES


