Antioxidant Potential of Morinda Lucida and Psidium Guajava Extracts and Actions Against Paracetamol-Induced Kidney and Liver Injuries in Rats

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Abstract
Antioxidant agents of plants origin have continued to attract interest because of the potential they hold in the maintenance of human health accompany with their minimal side effects. The present study sought to evaluate the comparative free radical scavenging activities of ethanol extracts of air dried Morinda lucida leaves (EMLL) and Psidium guajava leaves (EPGL) by measuring their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, nitric oxide (NO) radical, 2,2 azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS*), and inhibit lipid peroxidation (LPO). Antioxidant activities of the extracts were also determined in the plasma of the rats fed with the extracts by assaying for antiradical activity against DPPH and NO radicals in vitro. In vivo antioxidant effects of the extracts were also evaluated in paracetamol treated rats. Twenty rats were randomly divided into four groups for this study. Group 1 received normal feed as control, group 2 received 14.30mg/kg b.w of paracetamol by gavage, groups 3 and 4 received 400mg/kg b.w of EMLL and EPGL each for 7 days plus paracetamol on the 8th day respectively. Catalase (CAT) and superoxide dismutase (SOD) activities and malondialdehyde (MDA) status were assayed for in the kidney, liver and serum. Histopathological examinations of liver and kidney were also carried out. The results showed that EMLL and EPGL exhibited free radical scavenging ability in dose dependent manner towards DPPH, NO, ABTS radicals as well as inhibition of LPO. The results of evaluation of the antioxidant potentials of the extracts while in the plasma showed that they were associated with free radical scavenging activity in vivo. Paracetamol treatment caused significant (p<0.05) decreases in SOD and CAT activities, and marked increase (p<0.05) in MDA levels when compared with the control. However, compared with paracetamol only group, the extracts caused significant (p<0.05) increase in SOD and CAT activities and decreased MDA levels. Histopathological analysis of kidney and liver showed that the extracts were able to offer protection against paracetamol-induced kidney and liver injuries. The extracts therefore have strong antioxidant and cytoprotection abilities.

Keywords: Antioxidant, scavenge, antiradical activity, cytoprotection, injuries and treatment

1. Introduction
Historically, medicinal plants are used in the cellular and metabolic diseases treatment such as diabetes, obesity, cardiovascular diseases, and cancer etc. It is well known that the reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxides are highly reactive and potentially damaging transient chemical species (Mantle et al., 2000). The tissue damage results from an imbalance between ROS-generating and scavenging systems inside the body. The decrease or damage scavenging systems leads to a variety of disorders including degenerative disorders of the central nervous system (CNS) such as Alzheimer’s disease, cancer, atherosclerosis, diabetes mellitus, hypertension, AIDS, and aging (Halliwell and Gutteridge, 1998; Mantle et al., 2000).
In recent years, there has been a considerable interest in the finding of natural antioxidants from plant materials because synthetic antioxidants have been questioned due to their toxicity, (Valentao et al., 2002). It is reported that the phytochemicals from plants, particularly flavonoids and other polyphenols have been involved in antioxidant or scavenging of free radical reactions (Kinsella et al., 1993). Natural antioxidants could prevent the ROS related disorders in human beings without the use of synthetic compounds, which may be carcinogenic and harmful to the lungs and liver (Branen, 1986). Also, antioxidants play an important role in nutrition by lengthening the shelf life of food and the reducing nutritional losses (Grice, 1986; Wichi, 1988).

One commonly used medicinal plant is *Psidium guajava* Linn (Family Myrtaceae). It is commonly called guava, goyave, or goyavier in French, Guave, guayebaum and guayaus in German and guava in English (Glutierrez et al., 2008). *Psidium guajava* has been reported to possess anti-bacterial, anti-diarrheal, anti-hyperglycemic, antimalarial, anti-cytotoxic and antioxidants activities (Roy et al., 2006). Other medicinal uses of *Psidium guajava* are its antioxidant activities which protect the human body against, free radicals during oxidative stress. *Psidium guajava* possess antioxidant activity which performs a characteristic role in inhibition of super oxide radical production, hydroxyl radical scavenging activity, nitric oxide radical scavenging and inhibition of lipid peroxide formation. Different parts of guajava plants are used for medicinal purpose and the useful parts include; leaf, fruit and bark (Smith and Nigel, 1992).

*Morinda lucida* belongs to the Riubiaceae family. This is another medicinal plant in a tropical West Africa rainforest, also called Brimstone tree. In South West of Nigeria, it is locally called Oruwo while in Ghana it is known as Twi, konkroma or Ewe amake. Among the Togolese the plant is popularly called Ewe amaker or Atak ake (Bakare, 2007). Different parts of the plant are attributed with diverse therapeutic benefits. *Morinda lucida* leaf extract was reported to possess medicinal properties such as trypanocidal, antimalarial activities in rats (Raji et al., 2005). Many Southern Nigerians treat malaria by drinking aqueous leaf extract as a therapeutic benefit with no known adverse effect among users. Among the Yoruba herbalist (South-West Nigeria) fresh leaves of the plant is often macerated in palm-wine and its bitter decoction is used in the oral treatment of suspected diabetic patients usually for a few days (Adeneye and Agbaje 2008).

In the present study, an attempt has been made to evaluate the antioxidant activities, *in-vitro* and *in vivo* of ethanolic extracts of *M. lucida* and *P. guajava* in paracetamol induced toxicity.

2. Materials and Methods

2. Reagents and Chemicals

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Hydrogen peroxide, Potassium ferricyanide, sodium carbonate, butanol, methanol, acetic acid, thiobarbituric acid, sodium dodecyl acrylamide sulphate, Iron ii tetraoxosulphate vi used were a product of Sigma- Aldrich, USA. 1, 10-phenanthroline used were products of Merck, Germany.

**Collection of plant materials:** The leaves of *Psidium guajava* and *Morinda lucida*, were collected from the forest around Adekunle Ajasin University Akungba Akoko (AAUA), Ondo-State, Nigeria. The plants were identified in the Department of Plant Science and Biotechnology, AAUA.

2.2 Extraction Procedure

Cold extraction method was employed. 500g of the clean, air dried and pulverized plants samples were weighed differently into extraction jars respectively and 1400ml of analytical grade ethanol was added to the jars containing *Psidium guajava* and *Morinda lucida* respectively. The extraction mixtures were given constant agitation and were left for 72 hours. The supernatants were decanted separately and concentrated using a rotary evaporator at 40°C and the extracts were freeze dried. The extracts were packed inside an airtight sample bottle and kept at 4°C inside refrigerator until required for various *in-vitro* and *in vivo* antioxidant assessments.

2.3 Biochemical In-Vitro Assays

**DPPH Radical Scavenging Assay**

The *in-vitro* antioxidant activity of the sample was determined according to the method described by Mensor et al., (2001). To 1ml of plant extract, 1ml of ethanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (0.3mM) was added. The mixture was incubated in the dark for 30min. The assay was standardized with Tannic acid. The absorbance of the yellow colour solution was read at 517nm on a spectrophotometer using methanol as blank.

DPPH scavenged (%) = \((A \text{ control} - A \text{ sample}) / A \text{ control} \times 100\)

Where A control = the absorbance of the ethanol, A sample = the absorbance of the reaction mixture.
Nitric Oxide Radical Scavenging Assay
The *in-vitro* nitric oxide scavenging activity was estimated according to the method of Marcocci *et al.*, 1994. To 1ml sample, 1ml of sodium nitroprusside (10mM, aqueous) and 1 ml buffer (sodium phosphate buffer, 0.2M) were added. The mixture was incubated at room temperature for 150 min. this is followed by the addition of 0.1ml Griess reagent. The absorbance of the pink colour solution was read at 540nm on a spectrophotometer. The reaction was standardized with ascorbic acid. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-naphthyl ethylene diamine dihydrochloride (NED) was measured spectrophotometrically at 540nm. The *in-vitro* NO$^-$ scavenging activity of the sample was calculated by using the following formula:

Nitric oxide scavenging activity (\%) = \((A \text{ control} - A \text{ sample})/ A \text{ control} \times 100\)

Where \(A \text{ control} = \) the absorbance of the reaction mixture in the absence of sample.
\(A \text{ sample} = \) the absorbance of the reaction mixture.

ABTS$^*$ Radical Scavenging Activity Assay
The *in-vitro* ABTS$^*$ scavenging activity of the polyphenolics was determined according to the method of Re *et al.*, 1999. The stock solutions were of 8mM ABTS and 3mM Potassium persulphate. The working solution was then prepared by mixing the two stock solutions in equal quantity and allowing them to react for 12 hours at room temperature in the dark. To 1ml sample (1mg/ml), 1ml of ABTS was added. The absorbance was read at 734nm on a spectrophotometer.
Trolox was used as standard. The *in-vitro* ABTS$^*$ radical scavenging activity of the sample was calculated by using the following formula:

ABTS$^*$ scavenging activity (\%) = \((A \text{ control} - A \text{ sample})/ A \text{ control} \times 100\)

Where \(A \text{ control} = \) the absorbance of the reaction mixture in the absence of sample.
\(A \text{ sample} = \) the absorbance of the reaction mixture.

Inhibition of Lipid Peroxidation Assay
*In-vitro* inhibition of lipid peroxidation was estimated according to the method of Ruberto and Baratta, 2000. In this assay, egg yolk homogenate served as lipid rich medium, and FeSO$_4$ acts as initiator of lipid peroxidation. Briefly , 40 μl of plant extract was mixed with 0.25ml 10% egg yolk .This was followed by the addition of 10 ul FeSO$_4$(0.07 M, aqueous). The mixture was incubated at room temperature for 30min. This was followed by the addition of 0.75ml of glacial acetic acid (5%,v/v aqueous) and 0.75 ml of thiobarbituric acid 0.6% in 0.2 M NaOH .The mixture was incubated in a boiling water bath (90$^\circ$C) for 20min, cooled and centrifuged at 3000rpm. One milliliter (1 ml) of the pink colour supernatant was read at 532 nm on a spectrophotometer. The assay was standardized using Quercetin.

*In-vitro* inhibition of lipid peroxidation = \((A \text{ contol} – A \text{ sample})/A \text{ control} \times 100\).

Where \(A \text{ control} = \) absorbance of the control, \(A \text{ sample} = \) the absorbance of the sample.

Estimation of Malondialdehyde (MDA) Status
The assay method of Buege and Aust, 1998 was adopted.

**Principle**
MDA which is formed from the breakdown of polyunsaturated fatty acid serves as a convenient index for the determination of the extent of lipid peroxidation (LPO). MDA reacts with thiobarbituric acid to give a red product absorbing at 535nm.

**Procedure**
To 1 ml sample, 2 ml of TBA-TCA-HCL Reagent (ml) was added, and 3 ml of the reagent was added to the blank test tube. The contents of each test-tube was heated for 15 minutes in boiling water. After cooling, each tube was centrifuged at 4000rpm for 10 minutes to remove flocculent precipitates. Absorbance of each supernatant was read at 535nm against the blank.

**Estimation of Catalase (CAT) Activity**
This was determined according to the method of Cohen *et al.*, 1970.
**Principle**
Catalase catalyses the breakdown of hydrogen peroxide introduced in the presence of phosphate buffer to water and oxygen.

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

The reaction is thereafter quenched (stopped) by the addition of H$_2$SO$_4$. The amount of H$_2$O$_2$ remaining in the reaction mixture after few minutes of catalase action will be determined by titration with potassium permanganate (KMNO$_4$), a very strong oxidizing reagent. The amount of substrate remaining in the mixture is inversely proportional to the activity of the enzyme.

**Procedure**
To 0.5 ml of the sample, 5 ml of 30mM H$_2$O$_2$ was added while the blank tube contains 0.5 ml distilled water. The contents of the tubes were mixed and left standing for three minutes, 1.0 ml 6M H$_2$SO$_4$ was added to the sample, blank and standard tubes while 0.05 M phosphate buffer pH7.4 (for the standard tube) was added and thoroughly mixed by inversion and thereafter 7.0 ml 0.01 M KMNO$_4$ was added to all the tubes. Finally, the content of each tube was thoroughly mixed by inversion and the absorbance read at 480nm after 30-60 seconds against distilled water as blank. Catalase activity was calculated.

**Estimation of Superoxide Dismutase (SOD) Activity**
This was determined according to the method of Misra and Fridovich (1972).

**Principle**
Adrenaline auto-oxidizes rapidly in aqueous solution to aderenochrome, whose concentration can be determined at 420nm. The auto-oxidation of adrenaline depends on the presence of superoxide anions. Superoxide Dismutase catalyzes the breakdown of superoxide anions thus inhibiting the auto-oxidation of adrenaline. The degree of inhibition is thus a reflection of the activity of SOD and is determined as one unit of the enzyme activity.

**Procedure**
3 ml of the sample tubes contained 0.2 ml sample, 2.5 ml of 0.05M carbonate buffer pH10.2 and 0.3 ml of 0.03mM adrenaline. The blank tubes contained 3.0 ml of distilled water while the standard tubes contained 0.2 ml distilled water, 2.5 ml of 0.05M carbonate buffer pH10.2 and 0.3 ml of 0.03mM adrenaline. The content of each tube was mixed, and absorbance read at 420nm. SOD activity was calculated.

**Histological Test Preparations**
The liver and kidney were carefully removed and piece of the tissues were cut and kept in 10% formalin in carefully labeled universal containers for preservation prior to processing. The slides were then stained using the Haematoxylin-Eosin staining technique through increasing grade of alcohol and xylene before being mounted with a cover strip dried before viewing microscopically for possible malignant changes.

**3. Results**
Table 1. Showing DPPH free radical scavenging activity of plasma from rats treated with ethanolic extracts of M. lucida and P. guajava.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/ml)</th>
<th>DPPH (measured at 518nm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.649±0.064&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Normal</td>
<td>-</td>
<td>0.836±0.070&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.30</td>
</tr>
<tr>
<td>M. lucida</td>
<td>400</td>
<td>0.300±0.013&lt;sup&gt;c&lt;/sup&gt;</td>
<td>81.07</td>
</tr>
<tr>
<td>P. guajava</td>
<td>400</td>
<td>0.337±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79.56</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.364±0.004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77.73</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0.322±0.007&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80.47</td>
</tr>
</tbody>
</table>

Values with different superscript are significant different at p≤0.05, Mean±SD; n=3.

4. Discussions

Nitric oxide is an essential bioregulatory molecule required for some physiological processes like neural signal transmissions, immune response, control vasodilation and control of blood pressure (Palmer et al., 1987 and Gold et al., 1990). Nitric oxide has an important role in various inflammatory processes. Excessive production of this radical is directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetis, multiple sclerosis, arthritis and ulcerative colitis (Tylor et al., 1997). The toxicity of NO<sup>−</sup> increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO<sup>−</sup>) (Hue and Padjama, 1993). The nitric oxide generated by sodium nitroprusside reacts with oxygen to form nitrite. The extracts inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide. Figure 1 illustrates the % inhibition of nitric oxide generation by the extracts P. guajava, M. lucida and Ascorbic acid standard in concentrations 25, 50, 100, 200, and 400 µg/ml which significantly scavenged in dose dependent manner with an
IC$_{50}$ of 56.8 µg/ml by *M. lucida*, 53.0 µg/ml by *P. guajava* and 45.8 µg/ml by Ascorbic acid. *P. guajava* and *M. lucida* exhibited the inhibition of nitric oxide formation in vitro comparable with the ascorbic acid standard p<0.05. The ABTS- (2,2'-azinobis-3-ethyl-benzothiazoline-6-sulphonic Acid) radical reactions involve electrons transfer and the process take place at faster rate when compared to DPPH$^*$ radicals. Re *et al*., 1999 reported that the decolouration of the ABTS$^*$ radicals also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. In the ABTS$^*$ radical cation scavenging activity, the extracts showed concentration dependent scavenging activity. The percentage inhibition was observed to be concentration dependent. The IC$_{50}$ of the extracts were 26.5 µg/ml *P. guajava*, 171.96 µg/ml *M. lucida* and 28.10 µg/ml Quercetin standard. *M. lucida* exhibited a weak inhibition of ABTS$^*$ radical when compared with the standard quercetin. *P. guajava* had a comparable inhibition with standard quercetin at p<0.05.

1, 1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances. In this model it was observed that the tested extracts showed scavenging ability of DPPH$^*$ radicals in dose dependent manner with IC$_{50}$ values of 13.33 µg/ml *M. lucida*, 21.12 µg/ml *P. guajava*, and 90.99 µg/ml Tannic acid as standard. All the extracts showed better scavenging activity when compared with the standard p<0.05 with *M. lucida* showing the highest inhibition.

Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of ferrous sulphate. Lipid peroxides are likely involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging (Ames *et al*., 1993). The effects of extracts on non-enzymatic peroxidation are shown in graph above. All the tested extracts inhibited lipid peroxidation in a concentration dependent manner. IC$_{50}$ values for the inhibition of lipid peroxide formation were 37.5 µg/ml for *M. lucida*, 40.0 µg/ml for *P. guajava* and 77.5 µg/ml for Quercetin standard. So the extracts prevented lipid peroxidation better than the standard quercetin. The results suggest that consumption of these extracts may afford a cytoprotective effect.

It is known that paracetamol (PCM) induces liver injury through the action of its toxic metabolite, N-acetyl-benzoquinoneimine, produced by the action of Cytochrome P-450. This metabolite reacts with reduced glutathione (GSH) to yield non-toxic 3-GS-ylparacetamol. Depletion of GSH causes the remaining quinone to bind to cellular macromolecules leading to cell death (Udem *et al*., 1997). Damage induced in the liver is accompanied by the increase in the activity of some serum enzymes. Drugs having antioxidant activity are also effective in treating paracetamol induced hepatotoxicity by scavenging the free radicals produced by PCM metabolism, thereby preventing the liver damage induced by both PCM metabolite and due to depletion of glutathione. *Psidium guajava* is a known antioxidant (Qian and Nihorimbere, 2004) and this activity may be responsible for its protective effect in PCM induced hepatotoxic model.

Oxidative stress is frequently being used as a biomarker of the effect of exposure to pro-oxidant or toxicant (Adaipoh *et al*., 2007; Valavanidis, *et al*., 2006). One of the most damaging effects of reactive oxygen species and their products in cell is the peroxidation of membrane lipids, which can be measured by the amount of MDA formed (Li *et al*., 2008; Zerri *et al*., 2012). Biomarkers of oxidative stress, such as changes in antioxidant enzymes activity or in degree of accumulation of damaged molecules can offer an early warning sign for exposure to redox-active xenobiotics (Almroth, 2008).

The decrease values of MDA formed in groups treated with extracts plus paracetamol when compared with paracetamol treated only group showed that the extracts were able to offer protection against cytotoxicity presented by the paracetamol. In the serum, kidney and liver tissues of rats treated with paracetamol only, there were profound injuries to the tissues as seen from the histopathology results. However, the damage was ameliorated in the groups treated with extracts plus paracetamol. This implied that the extracts were able to show protective effect possibly by the process of antioxidant due to the presence of phenolic compounds present in them.

Catalase is a phase II enzyme involved in the conversion of hydrogen peroxide to water and oxygen. In this study, the level of catalase activity was low in the liver of rats treated with paracetamol only, and *M. lucida* plus paracetamol treated groups when compared with the *P. guajava* extract treated group p<0.05. The reduction could be due to the depletion of the enzymes following excessive ROS generated by the paracetamol. *M. lucida* may have slow rate of inducing or not even inducing the synthesis of catalase in the presence of the pro-oxidant. *P. guajava* was able to counter the oxidative stress posed by the paracetamol when compared with the untreated control group p<0.05. However, in the kidney, activity of catalase was not significantly different from the other groups except *M. lucida* group with corresponding low activity of catalase both in the liver and kidney. Catalase an antioxidant enzyme well distributed in many tissues helps to decompose hydrogen peroxide and offers protection to tissue from highly reactive hydroxyl radical (Chance and Greenstein, 1992). Superoxide and hydrogen peroxide were reversed in group treated with the *P. guajava* extract indicating protective ability of the plants. The anti-hepatotoxic action of
the ethanolic extracts (200mg/kg, p.o.) was substantiated by significant attenuation of the increased levels of serum enzymes in rats intoxicated with PCM (Chanchal and Amit, 2010).

SOD will scavenge superoxide anions which if allowed to accumulate will inhibit the activity of CAT (Mantha et al., 1996). The product of the dismutation activity of SOD is hydrogen peroxide which is a substrate for CAT. Thus, a decrease in SOD activity leads to a decrease in CAT activity for hydrogen peroxide degradation. PCM administration induced a collapse of the liver antioxidant defense system by inducing a decrease in the antioxidant enzymes activities. Similar results on PCM induced collapse of the antioxidant defense had earlier been reported (Uma et al., 2010; Sabina et al., 2013; Dash et al., 2007; Sowemimo et al., 2007). This effect of PCM was well tolerated by experimental animals receiving M. lucida hence, preventing the collapse of the antioxidant enzymes SOD and CAT (Fogha et al., 2015). The observed increase of SOD activity suggests that the methanolic extract of M. lucida leaf has an efficient protective mechanism in response to oxidative stress and may be associated with decreased oxidative stress and free radical-mediated tissue damage.

Super oxide dismutase (SOD) is associated with increase in ROS which arises from adverse metabolic activities such as lipid peroxidation. Activity of SOD was significantly low both in kidney and liver tissues of paracetamol treated group when compared with the control p<0.05. Also, this could be as a result of depletion of the enzymes because of ROS generated by the paracetamol. The extracts were able to induce the production of the enzyme to the level of scavenging the radicals generated by the paracetamol and ensure protection of the cells from damaging. Only M. lucida had unusually high (about 2-fold) values of the enzyme in the serum of the animals. May be the enzymes were mobilized from another point of synthesis to the affected tissues, as against the impression of leakage of maker enzymes in serum a general index of hepatic cytotoxicity (Tseng et al., 1997). Histopathology readings showed that paracetamol administration induced hepatocyte necrosis in localized areas of the liver with attendant provoking heavy acute inflammatory cells (polymorphs) infiltration. However, administration of paracetamol with extracts of M. lucida and P. guajava afforded appreciable protection to the hepatocytes. In the kidney, paracetamol administration induced acute tubular necrosis in patchy areas seen as cloudy swelling of tubular epithelial cells narrowing the lumel to slits. Paracetamol administration with the extracts provided appreciable protection to the nephrons.

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