Protective Effect of Moringa oleifera Lam. and Lankea kerstingii Extracts Against Cadmium and Ethanol-induced Lipid Peroxidation


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Abstract: The present study had evaluated the protective effect of hydroalcoholic (50-50: v/v) and aqueous extracts of L. kerstingii and M. oleifera against lipid peroxidation induced in vivo and in vitro by either cadmium or ethanol. In a first series of experiments, lipid peroxidation induced in vitro by cadmium (5 μg mL⁻¹) is decreased by hydroalcoholic extracts of M. oleifera and L. kerstingii (100 μg mL⁻¹) by 94% and 50% (p<0.001) respectively whereas their aqueous extracts (100 μg mL⁻¹) reduced the cadmium induced lipid peroxidation by 94% (p<0.001) and 44% (p<0.001) respectively. In vivo, the pretreatment with hydroalcoholic extracts of M. oleifera and L. kerstingii at 1 g kg⁻¹ b.wt. reduced significantly ethanol-induced lipid peroxidation, in liver, by 53 and 50% (p<0.001), respectively. Similar results were found in the kidney even though lipid peroxidation is slightly increased by ethanol in this organ.

Keywords: Antioxidative effect, free radicals, Moringa oleifera, Lankea kerstingii, cytotoxicity

INTRODUCTION

Free radicals are responsible for lipid peroxidation and have received much attention recently in connection with a variety of pathologies such as cancer (Zhu et al., 2002).

Cadmium a very toxic and ubiquitous metal and ethanol (95%) are known to induce lipid peroxidation (Traore et al., 2000; Bashandy and Alhazza, 2008). It is also known that the most effective way to prevent oxidative damages remains the use of antioxidant substances or nutrients which can be found in fruits and vegetables (Keen et al., 2005; Njáyou et al., 2008).

Moringa oleifera Lam. (Moringaceae) and Lankea kerstingii Engl. and K. Krause (Anacardiaceae) are medicinal plants respectively used to treat asthma and anaemia. Pharmacological studies of extracts of M. oleifera have revealed several properties such as hypolipidaemic (Mehta et al., 2003), hypotensive (Faizi et al., 1998), anticancer (Costa-Lotufo et al., 2005) and hepatoprotective (Pari and Kumar, 2002). Lankea kerstingii has been studied for its trypaoncidial effect (Atawodi et al., 2003).

Based on the above-mentioned report, we have undertaken this work in order to study (1) the protective effect of M. oleifera leaves and L. kerstingii stem bark extracts against lipid peroxidation induced in mouse by ethanol and in Caco-2 cells by cadmium, (2) the cytotoxicity on Caco-2 cells and (3) the toxicity of extracts of M. oleifera leaves and L. kerstingii barks given orally to mice.

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MATERIALS AND METHODS

Chemicals
Dulbecco’s Modified Eagle Medium (DMEM), Foetal Calf Serum (FCS), Ethylenediamine Tetraacetic Acid (EDTA), Phosphate-Buffered Saline (PBS), alpha-tocopherol (vitamin E) and trypsin-EDTA mixture were purchased from Sigma-Aldrich (Lyon, France). All other chemicals used were of analytical grade.

Plant Materials
Moringa oleifera leaves and Lannea kerstingii barks were collected locally from rural areas close to Lome (Togo) in September 2005. They were identified by the Department of Botany, University of Lome. A specimen of each plant was also deposited in the herbarium of the Department of Botany. The extraction was conducted in Animal Physiology Department, Faculty of Sciences, University of Lome, Togo.

Leaves and barks were shade dried and pulverised. The powder was extracted with water or ethanol-water (50:50 v/v). Dried powder was soaked in water and heated in bath water for 3 h (aqueous extract) or soaked in ethanol-water for 48 h (hydroalcoholic extract). Each extracted solution was filtered and evaporated by using a rotary evaporator. Moringa oleifera yielded respectively 18.67 and 21.47% for aqueous and hydroalcoholic extraction. Lannea kerstingii yielded 10.41% for the aqueous extract and 15.90% for the hydroalcoholic extract. Extracts were dissolved in double distilled water for final use.

Animals
Male and female mice (n = 38) weighting between 18 and 20 g were used for the in vivo experiments. The animals were purchased from the animal centre R. Janvier (France). They were housed in large cages in an environmentally controlled condition and they were fed on Standard Laboratory chow with water ad libitum and were acclimatized for a week before experiments. This study was conducted in Toxicology Department, Laboratory of Toxicology and Applied Hygiene, University Victor Segalen Bordeaux 2, France.

Cell Culture
The Caco-2 cells, a human colon cancer line, were obtained from Dr. Jing Yu, (Tufts School of medicine, Medford, Mass., USA). Cells were routinely cultured in a humidified 5% CO2, 95% air mixture at 37°C and were grown in DMEM medium, (Sigma, France), supplemented with 10% foetal bovine serum, 8 mM L-glutamine, penicillin (100 μl mL⁻¹) and streptomycin (100 μg ml⁻¹).

Induction of Lipid Peroxidation by Cadmium in Caco-2 Cells
Lipid peroxidation in Caco-2 cells was induced according to the method described by Mattias et al. (1999). Briefly, Caco-2 cells were cultured (1x10⁵ cells mL⁻¹) in 24 well non coated microplates, for 19 h at 37°C. Plant extracts (100, 200 and 300 μg mL⁻¹) and vitamin E (50 μg mL⁻¹) were added. After 24 h of contact, the supernatant was removed. Cadmium was added for 24 h. Then cells were trypsinised, centrifuged and resuspended in SET buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris-HCl, pH 8.0) for the determination of MDA level.

Induction of Lipid Peroxidation by Ethanol in Mouse Liver and Kidney after the Administration of Plant Extracts
The protective effect of hydroalcoholic extracts of M. oleifera and L. kerstingii on the liver and kidney lipid peroxidation was determined by the MDA-TBA adduct according to the modified method
described by Shieh et al. (2001). Briefly, the mice were divided into eight groups (n = 4): I (control); II (ethanol), III or IV, V or VI and VII or VIII (respectively, ethanol 95% + M. oleifera or L. kerstingii extract 1, 2 and 3 g kg^-1 b.w.t.). Groups III, IV, V, VI, VII and VIII, received orally 0.1 mL of ethanol 30 min after plants extract administration. Group I received only tap water and group II only ethanol. One hour after the treatment with ethanol, all mice were sacrificed under ether anaesthesia. The liver and kidney of each animal were excised, rinsed in ice cold saline to clear them of blood, weighed and homogenised in SET buffer (approximately 10% w/v) by using a Potter Elvehjem homogenizer with a Teflon pestle. Homogenates were then centrifuged at 600 g for 5 min at 4°C. One milliliter of each supernatant was centrifuged at 10,000 g for 15 min at 4°C and final supernatants were used to determine lipid peroxidation.

Determination of MDA-TBA Adduct

Lipid peroxidation was measured by quantification of MDA-TBA adducts formed during incubation, as previously described by Traore et al. (2000) and related to the protein content of tissue homogenates. The protein content was determined through the use of Bradford (1976) method. The same method was applied to Caco-2 cells homogenates.

Neutral Red Uptake Assay

Caco-2 cells were seeded in 96-wells microplates 10000 cells 200 μL^-1 well^-1 and routinely cultured in a humidified incubator for 24 h. Cells were maintained in culture and exposed to plant extracts over a range of concentrations (50-500 μg mL^-1). After 72 h exposure to extracts, Neutral Red (NR) uptake test was performed according to the procedure described by Yusuq et al. (2005). Briefly, at the end of the treatment (72 h), the medium with or without extracts was discarded and 200 μL of freshly prepared neutral red solution (50 μg mL^-1) were added to each well. Cells were then re-incubated for an additional 4 h at 37°C. Thereafter, the cells were carefully washed twice with 200 μL of PBS to eliminate extracellular NR. The incorporated dye was eluted from the cells by adding 200 μL elution medium (50% ethanol supplemented with 1% acetic acid, v/v) to each well followed by gentle shaking of the microplate for 15 min. The plates were then read at 540 nm using a microplate reader (Dynatech MR 4000, Dynatech, Boston, MA, USA).

Survived cells in treated wells were expressed as percentage of control wells. The IC_{50} (50% viability inhibitory effect) was determined and expressed in μg mL^-1.

MTT Assay

This test was carried out according to the method described by Kouadio et al. (2005). Caco-2 cells were seeded in 96-wells microplates (10000 cells 200 μL well^-1) and routinely cultured in a humidified incubator for 24 h. Cell culture media were removed and extracts were added in concentration ranging from 50 to 500 μg mL^-1. Cells were then incubated for 72 h. In this test, a control group (DMEM without extract) and a blank group (without cells or medium) were also included. The medium with or without extract was then discarded and 100 μL of tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (0.5 mg mL^-1 in DMEM) were added to each well. Cells were re-incubated for an additional 2 h, 100 μL of 10% SDS in 0.01 M HCl were added to each well to dissolve the formazan crystals. The plates were then read on a microplate reader (DYNATECH MR 4000, Dynatech, Boston, MA, USA) at 560 nm. Four wells were used for each concentration of extract.

Acute Toxicity Test

The limit test dose of 5,000 mg kg^-1 was used as described by the Organization for Economic Cooperation Development (OECD, 2002) guideline. Three female mice each sequentially dosed at
interval of 48 h (short term observation period) were used for the test. Animals were observed individually for any sign of acute toxicity and behavioural changes 1 hour post dosing and at least once daily for 14 days.

**Statistical Analysis**

The results are expressed as Mean±SEM. Statistical analysis was performed by Analyse of Variance (ANOVA) followed by Fischer LSD test using Systat 5.0. Results were considered significant if the probability p<0.05.

**RESULTS**

Cadmium increased significantly (p<0.001) Caco-2 cells MDA level by 925% (Table 1) and ethanol increased the liver and kidney MDA level (Table 2), respectively by 48% and 16% (p<0.05) compared to control.

Pretreatment of Caco-2 cells with vitamin E (positive control), aqueous (100, 200 and 300 µg mL⁻¹) and hydroalcoholic extract (100, 200 and 300 µg mL⁻¹) of *M. oleifera* decreased MDA level in cadmium-treated cells. This effect did not appear concentration dependant. The treatment of Caco-2 cells with aqueous (100 µg mL⁻¹) and hydroalcoholic extract (100 µg mL⁻¹) of *L. kerstingii* also decreased significantly (p<0.001) cadmium-induced MDA production by 44% and 50%, respectively. At 200 µg mL⁻¹ and 300 µg mL⁻¹, *L. kerstingii* hydroalcoholic and aqueous extract was more effective (Table 1). Similarly to *M. oleifera* extracts this effect did not appear concentration dependant. *M. oleifera* and *L. kerstingii* administered orally showed a significant decrease (p<0.001) of MDA level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration (µg mL⁻¹)</th>
<th>MDA (pmones mg⁻¹ of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>361±52</td>
</tr>
<tr>
<td>Cd</td>
<td>5</td>
<td>3308±242**</td>
</tr>
<tr>
<td>Cd+Vitamin E</td>
<td>50</td>
<td>256±30**</td>
</tr>
<tr>
<td>Cd+hydroalcoholic extract of <em>L. kerstingii</em></td>
<td>100</td>
<td>1897±240**</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>580±172**</td>
</tr>
<tr>
<td>-</td>
<td>300</td>
<td>495±153**</td>
</tr>
<tr>
<td>Cd+aqueous extract of <em>L. kerstingii</em></td>
<td>100</td>
<td>1694±240**</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>422±143**</td>
</tr>
<tr>
<td>-</td>
<td>300</td>
<td>319±179**</td>
</tr>
<tr>
<td>Cd+hydroalcoholic extract of <em>M. oleifera</em></td>
<td>100</td>
<td>203±30**</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>208±3**</td>
</tr>
<tr>
<td>-</td>
<td>300</td>
<td>223±34**</td>
</tr>
<tr>
<td>Cd+aqueous extract of <em>M. oleifera</em></td>
<td>100</td>
<td>209±36**</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>252±5**</td>
</tr>
<tr>
<td>-</td>
<td>300</td>
<td>202±20**</td>
</tr>
</tbody>
</table>

Data are Mean±SEM. **Significantly different as compared to cadmium with p<0.001, ***Significantly different as compared to control with p<0.001.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (g kg⁻¹ body wt.)</th>
<th>MDA (pmones mg⁻¹ of protein) in liver</th>
<th>MDA (pmones mg⁻¹ of protein) in kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>2266±98</td>
<td>1960±65</td>
</tr>
<tr>
<td>Ethanol 95%</td>
<td>-</td>
<td>3556±133*</td>
<td>2268±105*</td>
</tr>
<tr>
<td>Ethanol + extract of <em>L. kerstingii</em></td>
<td>1</td>
<td>1562±17**</td>
<td>2000±167</td>
</tr>
<tr>
<td>-</td>
<td>2</td>
<td>1372±169**</td>
<td>1759±99**</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
<td>1453±85**</td>
<td>1659±60**</td>
</tr>
<tr>
<td>Ethanol + extract of <em>M. oleifera</em></td>
<td>1</td>
<td>1692±140**</td>
<td>2209±113</td>
</tr>
<tr>
<td>-</td>
<td>2</td>
<td>1252±164**</td>
<td>1571±91**</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
<td>1735±60**</td>
<td>2040±60**</td>
</tr>
</tbody>
</table>

Data are Mean±SEM. **Significantly different as compared to ethanol with p<0.001, *Significantly different as compared to control with p<0.001, **Significantly different as compared to control with p<0.001, ***Significantly different as compared to control with p<0.001.
Table 5: Effect of hydroalcoholic and aqueous extracts of *L. kerstingii* and *M. oleifera* on Caco-2 cells viability as measured by MTT assay and neutral red uptake

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MTT assay</th>
<th>Neutral red uptake assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroalcoholic extract of <em>L. kerstingii</em></td>
<td>186</td>
<td>328</td>
</tr>
<tr>
<td>Aqueous extract of <em>L. kerstingii</em></td>
<td>228</td>
<td>386</td>
</tr>
<tr>
<td>Hydroalcoholic extract of <em>M. oleifera</em></td>
<td>264</td>
<td>274</td>
</tr>
<tr>
<td>Aqueous extract of <em>M. oleifera</em></td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

in liver by 53% and 50% compared to ethanol-treated group. Similar results were obtained, in liver, when extracts were administered at either 2 or 3 g kg\(^{-1}\) b.wt. But in kidney, pretreatment with hydroalcoholic extract of *M. oleifera* and *L. kerstingii* at 1 g kg\(^{-1}\) body wt did not decrease the level of MDA (Table 2). Furthermore, the administration of *L. kerstingii* hydroalcoholic extract at 2 g kg\(^{-1}\) body wt and 3 g kg\(^{-1}\) body wt was more effective and decreased kidney MDA level respectively by 22% (p<0.05) and 26% (p<0.001). *M. oleifera* hydroalcoholic extract at 2 g kg\(^{-1}\) body wt reduced also the kidney MDA level by 31% (p<0.001). But, at 3 g kg\(^{-1}\) body wt, *M. oleifera* showed higher MDA production as compared to 2 g kg\(^{-1}\) body wt (Table 2).

The limit dose of 5 g kg\(^{-1}\) did not cause any mortality or any signs of acute toxicity in any of the three rats tested in the short term (i.e., 48 h) and long term (i.e., 14 days) observatory period. The LD\(_{50}\) of the extract according to OCDE guidelines is therefore greater than 5 g kg\(^{-1}\).

The hydroalcoholic extracts of *L. kerstingii* and *M. oleifera* were more efficient in reducing cell viability than their aqueous extracts (Tables). According to MTT assay *L. kerstingii* is the most cytotoxic exhibiting a lower IC\(_{50}\) value, but neutral red uptake assay showed that hydroalcoholic extract of *M. oleifera* has the lower IC\(_{50}\).

**DISCUSSION**

This study aimed to evaluate the protective effect of *M. oleifera* leaves and *L. kerstingii* stem bark extracts against lipid peroxidation induced in mouse by ethanol and in Caco-2 cells by cadmium. It has been demonstrated previously that cadmium and ethanol increase free radicals production (Shieh et al., 2001; Ognjanovic et al., 2003; Bashandy and Alhazza, 2008).

Several studies had demonstrated the protective effect of plant extracts against lipid peroxidation (Rahmat et al., 2004; Costu-Lotufo et al., 2005). This effect is due to the presence of some potent free oxygen radicals scavengers such as: polyphenol, terpenoid, sulphide, curcumin, sterol and vitamins in these extracts (Winston, 1999).

*Moringa oleifera* is known to be a source of antioxidants, because of its total phenolic (Bajpai et al., 2005), vitamin A (Nambar and Seshadri, 2001) and vitamin E (Ching and Mohamed, 2001) contents. In our experimental conditions, *M. oleifera* showed an *in vitro* antioxidant activity, comparable to vitamin E (50 µg mL\(^{-1}\)) which is known to reduce lipid peroxidation (Ognjanovic et al., 2003).

In comparison to *M. oleifera*, very little is known about *L. kerstingii*. In our study, *L. kerstingii* showed a remarkable protective effect on lipid peroxidation. These results are in line with previous data from Diallo et al. (2001) who reported that *Launaea velutina*, a species very close to *L. kerstingii*, has antioxidant and radical scavenging activities.

In kidney, the higher dose of *M. oleifera* tested (3 g kg\(^{-1}\) b.wt.) showed lower antioxidant activity as compared to 2 or 1 g kg\(^{-1}\) b.wt. This could be explained by the possible shift from antioxidant activity to pro oxidant activity when doses or concentrations are increased. Similar results are reported in the literature elsewhere (Tafazoli et al., 2005).

The result of the acute toxicity study indicated that the LD\(_{50}\) of the aqueous and hydroalcoholic extracts of *M. oleifera* leaves and *L. kerstingii* stem bark are more than 5,000 mg kg\(^{-1}\). The limit test is primarily use in situations where the investigator has information indicating that the test material is likely to be non-toxic or of low toxicity (OCDE, 2002). The result of the acute oral toxicity study
therefore suggest that the extracts of *M. oleifera* leaves and *L. kerstingii* stem bark at the limit dose tested are essentially non-toxic and safe in oral formulation. The Caco-2 cells viability test indicated that hydroalcoholic extracts were more cytotoxic than aqueous extracts and *L. kerstingii* appears more cytotoxic than *M. oleifera*.

These results were however obtained with quite large amount of plant extracts (50 μg mL⁻¹ and up to 500 μg mL⁻¹) due to overall low toxicity of these plants used in traditional medicine in Africa. Many studies had showed a cytotoxic activity of *M. oleifera* against tumour cell lines (Mehta *et al.*, 2003; Costa-lotufo *et al.*, 2005). The IC50 values obtained using MTT test were in HL-60, CEM, HCT-8 and B16 cells, 60, 12.7, 113.8 and 28.8 μg mL⁻¹ respectively. In our investigation, the IC50 values in MTT test were 284 μg mL⁻¹ for hydroalcoholic extract and over 500 μg mL⁻¹ for aqueous extract but Caco-2 cells are known to be very resistant to cytotoxic drugs (Kralj *et al.*, 2003).

The values of IC₅₀ were lower in MTT test than in NR test except the case of *M. oleifera*. These two viability tests do not measure the same parameters. The MTT test measures mitochondrial activity, while NR test measures the integrity of endosomes, lysosomes and membranes (Zhang *et al.*, 1990). Active substances of *L. kerstingii* may target mitochondria, while *M. oleifera* extract seemed to target membranes, endosome and lysosome and reticulum endoplasmic.

In the present experiment, pretreatment with *M. oleifera* and *L. kerstingii* reduced cadmium and ethanol induced lipid peroxidation, this effect was not dose related and it seemed that the lower doses or concentrations already display high effect. From the results mentioned above, it can be concluded that, the use of *M. oleifera* and *L. kerstingii* has the capability to alleviate many of harmful effects of cadmium and ethanol. The findings of both acute oral and cytotoxicity could be an indication that *M. oleifera* and *L. kerstingii* have some large level of safety margin in oral formulation. These results support at least partially the traditional use of *M. oleifera* and *L. kerstingii* in the treatment of many diseases. However, further investigations are needed to know the nature of the actives substances and mechanism triggering these protective effects.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


