IJPSR (2011), Vol. 2, Issue 6 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 February, 2011; received in revised form 25 April, 2011; accepted 05 May, 2011

ANTIOXIDANT, ORGAN PROTECTIVE AND AMELIORATIVE PROPERTIES OF METHANOL EXTRACT OF *ANOGEISSUS LEIOCARPUS* STEM BARK AGAINST CARBON TETRACHLORIDE- INDUCED LIVER INJURY

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Keywords:

Medicinal plant, antioxidant activity, oxidative stress, lipid peroxidation, Malondialdehyde , *Anogeissus leiocarpus*

Abbreviations:

MDA- Malondialdehyde; TBARS- Thiobarbituric Reactive Substances; ROS- Reactive Oxygen Species; TCA- Trichloroacetic acid; PBS- Phosphate Buffered Saline; WHO- World Health Organization

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ABSTRACT

The methanol extract of the stem bark of *Anogeissus leiocarpus* was evaluated *in vivo* at 2.5mg/kg for antioxidant, hepatoprotective and ameliorative effect on hepatocellular injury following pre-treatment or post-treatment with carbon tetrachloride (CCl_4). Levels of the lipid peroxidation product, malondialdehyde (MDA) or thiobarbituric reactive substances (TBARS) were taken as a measure of oxidative stress, while levels of biochemical such as aspartate aminotransferase, alanine aminotransferase and bilirubin (total and conjugated) were taken as biomarkers of liver damage. Irrespective of whether the animals were pre- or post- treated with *Anogeissus leiocarpus*, there was no statistical difference between the levels of MDA and markers of liver damage in the groups on *A. Leiocarpus* + CCL_4 group and Vitamin E + CCl4, although both were significantly ($P<0.05$) lower than in the group on CCL_4 only.

However, malondialdehyde (MDA) levels in the heart, and to some extent, in the kidney, were hardly influenced by the treatments. These results indicate that methanolic extract of the stem bark of *Anogeissus leiocarpus* possess antioxidant, hepatoprotective and ameliorative effect on hepatocellular injury which may in part, account for the mechanism by which this plant brings about some of its reported therapeutic effects.

INTRODUCTION: According to Allen and Tresini (2000), reactive oxygen species (ROS) such as superoxyl, hydroxyl and peroxyl radicals are produced as part of normal metabolic processes and are believed to be involved in the oxidative damage of lipids, proteins, nucleic acids and other macromolecules. This causes different chronic diseases which among others include cancer, atherosclerosis, ageing, diabetes, asthma and cardiovascular disorders (Hadi *et al*., 2000). In developing countries of Africa where about 200,000 to 300,000 known plant species are found, and where about 85% of the population relies on traditional medicine as the cornerstone of their healthcare delivery, the United Nations encourages the inclusion of herbal medicine of proven safety and efficacy in their healthcare delivery program (UNESCO, 1998; Ampofo, (1997).

Anogeissus leiocarpus is a plant that is widely used in Northern Nigeria ethnomedicine. It belongs to the phylum, Tracheophyta; Order; *Myrtales and* Family: *Combretaceae* (combretoideae). It is commonly called Axle-wood tree, and in Nigeria, it is referred to as Marke (Hausa), Kojoli (Fulani), Annum (Kanuri), Ayin or Orin-odan Ainy (Yoruba), Atara (Igbo) and Kukunchi (Nupe). It is a very graceful tropical tree which grows up to 28m and occurs in the most of the savannah

areas from the driest regions to the borders of the forest zone. In Africa, its occurrence extends from Senegal in West Africa to Sudan and Ethiopia in East Africa. Those growing in the driest area tend to have smaller leaves and more hairy flowers than those growing under wetter conditions, but both differences are not sufficiently marked to create distinct varieties (Abdullahi, *et al*., 2003).

In Nigeria, earlier workers have reported that *Anogeissus Leiocarpus* may serve as remedies for gonorrhea, diabetes, hypertension, general body pain, blood clotting agent, as acaricide and as antihelmentic in different communities (Abdullahi, *et al*, 2003; Agaie *et al.,* 2007). Our mini- ethnopharmacological survey in parts of Northern Nigeria in the preliminary stages of this work in 2006 revealed that the plants may also be useful in the treatment of asthma, cough, tuberculosis, diabetes, etc. Because some of these diseases may have ROS as their etiological origin, we investigated the plant, *Anogeissus Leiocarpus*, for its antioxidant capacity, utilizing measurement of malondialdehyde (MDA) in organs of rats pre- and post-treated with carbon tetrachloride. Possible hepatoprotective activity was also assessed using markers of liver damage like bilirubin (total and conjugated), alanine aminotransferase, aspartate aminotransferase, alkaline phophatase and protein

MATERIALS AND METHODS:

Reagents and Chemicals: All reagents and chemicals used were of analytical quality acquired from Serva (Heidelberg, Germany), Fluka Chemie *GmbH*. Buchs or Sigma Aldrich Sigma Aldrich Chemie GmbH, Steinheim, Germany.

Experimental Animals: Male albino rats weighing between 150 and 250g were used for the experiment. The animals were acquired from the Department of Pharmacology, Ahmadu Bello University, Zaria, Nigeria. All animals were allowed to acclimatize under the laboratory condition for at least two weeks before commencement of the experiments. They were maintained *ad libitum* on stranded rat pellets (ECWA, Bukuru-Jos, Nigeria) and water, but these were withdrawn 15 -18 hours prior to commencement of experiments.

Plant Collection and Identification: *Anogeissus leiocarpus* plant was collected from Samaru-Zaria in Kaduna State of Northern Nigerian in June 2007. The Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria confirmed the identity of the plant, and specimen with Voucher No. 1625 was deposited.

Sample Preparation and Extraction: The stem bark of the plant was collected, the thin outer layer was scrapped, and the bark cut into small sizes prior to drying, first at room temperature, and later in the hot air oven at 40^{o}C until brittle. Dried sample was pounded in laboratory mortar into fine particles. The powdered sample (50g) was first extracted with 300mL of petroleum ether on Soxhlet apparatus for 6 hrs, and then with two portions of 300ml each of methanol for 3hrs each. The two methanol extracts were combined and methanol expelled at reduced pressure using a rotavapor set at 40^{o}C. The weight of the extracts were recorded and then stored in a refrigerator at 4^{o}C until required.

Animal Groupings: To investigate the capacity of the extract to protect against oxidative stress, the animals were randomly divided into groups of six as follows: solvent only (0.15% Tween 80 in corn oil), vitamin E only, Vitamin E pre-treatment + CCl_4; *A. leiocarpus* only (2.5mg /Kg) and *A. leiocarpus* pre-treatment + CCl_4 and CCl_4 only. However to establish the ameliorative effect of *A. leiocarpus* on pre-existing oxidative stress condition, the following groupings were used: solvent only (0.15% Tween 80 in corn oil), *A. leiocarpus* only; and CCl_4 pre-treatment + *A. leiocarpus,* vitamin E only, and CCl_4 only. In the experiment designed to study the protective effect of the plant against oxidative stress, the animals were pre-treated with the extract for three days before administration of the carbon tetrachloride, which was administered one hour after the extract treatment on the third day. However, for study designed to study the ameliorative effect of the plant, carbon tetrachloride was first administered at 0.60ml/kg, and the extract was administered one hour later, and then daily for the next two consecutive days. All animals were sacrificed 24 hours following last administration of the drugs or extracts, and the blood and organs were harvested, processed and stored in

deep freezer at -20°C for analysis of TBARS and other biochemical parameters.

Preparation and Administration of Extract and Vitamin E: Dried methanolic extract of the bark of *A. leiocarpus* (20 mg) was first made into a paste using 300 µl of Tween 80 warmed to about 40 °C, and then I.7 ml of corn oil was stirred vigorously into it to make a solution of 10 mg/ml). This was administered intraperitoneally to rats to achieve the desired dose of 2.5 mg/kg for two consecutive days Vitamin E (as tocopherol acetate gel; Health Aid Ltd, Middlesex, UK) was used as the standard antioxidant. It was appropriately diluted in corn oil (20% v/v; 134 mg/ml) and administered as single intraperitoneal dose of 50mg/kg for studies on the ameliorative effect against oxidative stress and to deliver same dose for two consecutive days for studies on the protective effect of the extract.

Animal Sacrifice and Blood/Organ Collection: Twenty four hours after the last administration of standard or test compound, the rats were sacrificed by decapitation following mild chloroform anesthesia, and the blood of each animal was separately collected into clean dry test tube, allowed to clot, and serum collected after about 3 hrs, following centrifugation at 3000 rpm for 15 mins. Each animal was then dissected and the liver collected immediately with rinsing in double portions of ice-cold normal saline, weighed, kept in separate transparent polythene bags and stored in a deep freezer at -20°C until analysis, which was usually within 24 hrs.

Tissue Homogenization: Each organ was homogenized in ice-cold phosphate buffer (pH7.2) so as to provide a 10% homogenate solution. These homogenates were centrifuged at 3000 g for 10 minutes to obtain supernatants that were immediately stored in deep freezers at -20°C until required for analysis, usually within 24 hrs for tissue malondialdehyde (MDA) content. The level of protein in the homogenate was determined using the Biuret method.

Assay for Thiobarbituric Acid Reactive Substances (TBARS): Oxidative damage was assessed spectrophotometrically by measuring the levels of malondialdehyde produced by lipid peroxidation as thiobarbituric acid reactive substances (TBARS) when reacted with 2-thiobarbituric acid solution (Yavuz et al, 2004; Sivonova *et al*, 2007). For this purpose, 100 µL of the supernatant was deproteinized by adding 2 ml of 14% trichloroacetic acid (TCA) and 2 ml of 0.67% thiobarbituric acid solution. The mixture was heated in a water bath at 80°C for 30 minutes to complete the reaction and then cooled rapidly on ice for 5 minutes. After centrifugation at 2000 g for 10 minutes, the absorbance of the colored product (TBARS) was measured at 532 nm with a UV spectrophotometer. The concentrations of TBARS were determined in triplicate and calculated using the molar extinction coefficient of malondialdehyde -1.56×10^5 mol/L/cm (Yavuz *et al*, 2004 and Sivonova *et al*, 2007). All TBARS concentrations are expressed in µmol/g tissue protein.

Bilirubin Estimation: Total and direct bilirubin were assayed as per manufacturer's instruction using a test kit (Human Geselschaft for Biochemica and Diagnostica GmbH, Wiesbaden, Germany) that is based on reaction of bilirubin with diazotized sulphanilic acid to form an azo dye that is measured at 546 nm.

Data Analysis: Data generated were subjected to one way analysis of variance (ANOVA). When the ANOVA showed that there differences in the mean values among the treatment groups are greater than would be expected by chance, it was then subjected to Tukey Test (multiple comparison procedures) to establish the statistically significant groups.

RESULTS:

Protective effect of *A. leiocarpus on* CCl4-induced oxidative stress and Liver injury: For the assay of malondialdehyde (MDA) in the liver, the levels were significantly higher ($P<0.05$) in the group in CCL_4 only than in the solvent control. Also, there was a significant difference between the levels in the CCL_4 only group and vitamin E only group. However there was no such statistical difference between the *A. Leiocarpus* + CCL_4 group and Vitamin E pre-treatment with CCl_4, although both were significantly lower than in the group on CCL_4 only. Similarly there was no statistical difference between the levels on Vitamin E + CCL_4 and Vitamin E only, although both were significantly lower than CCL_4 only (**Table 1**).

TABLE 1: EFFECT OF *A. LEIOCARPUS* PRE-TREATMENT ON LEVELS OF MALONDIALDEHYDE (MDA) IN LIVER, KIDNEY AND HEART OF CCL_4 - TREATED RATS (MEAN ± STD)

Treatment	Liver MDA (µmol/g)	Kidney MDA (µmol/g)	Heart MDA (µmol/g)
Solvent control	0.906± 0.087 [a]	0.216 ±0.038 [a]	0.158 ±0.067 [a]
CCl_4 only	2.750 ± 0.189 [c]	0.753 ±0.066 [c]	0.245 ±0.056 [b]
Vitamin E only	1.064 ± 0.092 [a]	0.218 ±0.028 [a]	0.176 ±0.019 [a]
Vitamin E + CCl_4	1.486 ± 0.151 [b]	0.308 ±0.049 [b]	0.238 ±0.062 [b]
A. leiocarpus only	0.979 ± 0.148 [a]	0.256 ±0.036 [a]	0.168 ±0.082 [a]
CCl_4 + *A. leiocarpus*	1.336 ± 0.200 [b]	0.734 ±0.142 [c]	0.179 ±0.064 [a]

Results with different superscripts along the vertical columns are statistically significant (P<0.005)

Also, the levels of markers of liver damage (total bilirubin, direct bilirubin, alanine aminotransferase and aspartate aminotransferase) were significantly higher in CCl_4 only (P<0.05) than other groups, including, *A. leiocarpus* + CCl_4 group. But like in the case of MDA levels, no such statistical difference existed either between Vitamin E only and Vitamin E + CCl_4 nor *A. leiocarpus* only and *A. leiocarpus* + CCl_4, even for the markers of liver damage (**Table 2**).

TABLE 2: EFFECT OF CCL_4 PRE-TREATMENT ON LEVELS OF MALONDIALDEHYDE (MDA) IN LIVER, KIDNEY AND HEART OF *A. LEIOCARPUS*-TREATED RATS (MEAN ± STD)

Treatment	Liver MDA (µmol/g)	Kidney MDA (µmol/g)	Heart MDA (µmol/g)
Solvent control	0.986± 0.075 [a]	0.269 ±0.088 [a]	0.163 ±0.097 [a]
CCl_4 only	2.890 ± 0.211 [b]	0.772 ±0.102 [b]	0.255 ±0.048 [b]
Vitamin E only	1.089 ± 0.142 [a]	0.206 ±0.086 [a]	0.174 ±0.016 [a]
CCl_4 + Vitamin E	2.377 ± 0.150 [c]	0.286 ± 0.070[a]	0.223 ± 0.040[b]
A. leiocarpus only	0.987 ± 0.132 [a]	0.247 ±0.084 [a]	0.172 ±0.089 [a]
CCl_4 + *A. leiocarpus*	2.298 ± 0.212 [c]	0.330 ± 0.090[c]	0.184 ± 0.041[a]

Results with different superscripts along the vertical columns are statistically significant (P<0.005)

Ameliorative effect of *A. leiocarpus on* CCl_4-induced oxidative stress and liver injury: Whereas, the MDA levels in the liver of animals in CCl_4 groups were significantly higher (P<0.05) than that of solvent only group, no much difference was observed between the MDA levels in CCl_4 +A. *leiocarpus* and CCl_4 + Vitamin E were not significantly different from that of solvent control (**Table 3**). A similar pattern was observed for the total bilirubin, direct bilirubin levels, alanine aminotransferase and aspartate aminotransferase (**Table 4**). However, the level of reduction of markers of oxidative stress and liver damage was much lower in the ameliorative experiment (Table 4) than in the preventive experiment (Table 3).

TABLE 3: EFFECT OF *A. LEIOCARPUS* PRE-TREATMENT ON LEVELS OF LIVER FUNCTION PARAMETERS OF CCL_4- TREATED RATS (MEAN ± STD)

Treatment	ALT (U/L)	AST (U/L)	Total Bilirubin (µmol/L)	Conjugated Bilirubin (µmol/L)
Solvent control	49.3 ± 9.2 [a]	93.10 ± 11.5 [a]	5.779± 0.941 [a]	1.927 ±0.158 [a]
CCl_4 only	104.5 ± 16.5[c]	140.4 ± 18.2 [c]	21.637±2.312 [c]	6.373±0.378 [c]
Vitamin E only	42.6 ± 7.4 [a]	81.2 ±15.0 [a]	5.558 ±0.890 [a]	1.853 ±0.105[a]
Vitamin E + CCl_4	82.3 ± 10.4[b]	109.3± 9.1 [b]	10.448±1.231 [b]	3.335 ±0.443 [b]
A. leiocarpus only	51.8 ± 8.5[a]	89.0 ±10.5 [a]	5.242 ±0.882 [a]	1.890 ±0.291 [a]
CCl_4 + *A. leiocarpus*	82.6 ± 11.2[b]	110.6± 15.2 [b]	10.393±1.735 [b]	3.223 ±0.302 [b]

Results with different superscripts along the vertical columns are statistically significant (P<0.005)

TABLE 4: EFFECT OF CCL_4 PRE-TREATMENT ON LEVELS OF LIVER FUNCTION PARAMETERS IN *A. LEIOCARPUS*-TREATED RATS (MEAN ± STD)

Treatment	ALT (U/L)	AST (U/L)	Total Bilirubin (µmol/L)	Conjugated Bilirubin (µmol/L)
Solvent control	79.2 ± 23.4 [a]	104.5 ± 11.0 [a]	5.793 ±1.041[a]	1.926 ± 0.098 [a]
CCl_4 only	204.5± 35.3[c]	203.7 ± 19.5 [c]	25.257±3.312[c]	6.373± 0.218 [c]
Vitamin E only	67.6±14.0[a]	101.2 ±11.5 [a]	5.617 ±0.490 [a]	1.853 ±0.115[a]
CCl_4 + Vitamin E	135.7± 21.5[b]	158 ± 15.4[b]	12.227 ± 0.120[b]	4.203 ± 0.090 [b]
A. leiocarpus only	61.3± 9.2[a]	99.8± 13.6 [a]	5.258 ± 0.682[a]	1.890 ± 0.091 [a]
CCl_4 + *A. leiocarpus*	134.5 ± 29.2[b]	146.4 ±12.5[b]	12.893 ± 1.25[b]	4.001 ± 0.102 [b]

Results with different superscripts along the vertical columns are statistically significant (P<0.005)

DISCUSSION: According to Johnston (1999), the levels of bilirubin can be used to assess liver function: when only conjugated (direct) bilirubin is elevated, the cause of the elevated bilirubin in the system is not from the liver, but when total bilirubin level is elevated, then it can be concluded that the elevated bilirubin level is due to liver damage. Thus, that the level of total bilirubin was higher in the 'CCl_4 only' group than all the other groups, confirms the damage to the liver by CCl_4. This damage was further reflected in the levels of other markers of liver damage, namely aspartate aminotransferase and alanine aminotransferase (Tables 3 and 4). That pre-treatment or post-treatment with either vitamin E or *Anogeissus leiocarpus* extract markedly lowered their levels compared to those treated with CCl4 only, strongly indicate that the plant can prevent and, to some extent, ameliorate liver damage. It is interesting to note that although to a lesser extent, difference also existed between the malondialdehyde levels in the extract-treated groups and CCl_4 –treated groups for the kidney and the heart, suggesting that though CCl4 is not directly a nephrotoxic or cardiotoxic agent, severe oxidative stress in the liver, may influence oxidative status of the kidney and heart.

That there was no statistical difference between the levels of markers of oxidative damage in groups on *A. Leiocarpus* + CCL_4 group and Vitamin E pre-treatment with CCl_4, although both were significantly lower than levels in the group on CCL_4 only, which were highly elevated (Table 1 and 2), indicate that administered CCl_4 could cause extensive oxidative and hepatocellular damage which can be prevented through pre-treatment with either Vitamin E or *A. leiocarpus* bark methanolic extract. Thus, *A. Leiocarpus* significantly provided protection against lipid peroxidation just like Vitamin E. This is consistent with previous reports which elaborated on the role of antioxidant vitamins, minerals, drugs and plant-derived compounds in the prevention and therapy of liver fibrosis, and concluded that an increased liver content of vitamin E leads to a significant degree of protection against carbon tetra chloride – induced chronic liver damage and cirrhosis in rats (Parola *et al*, 1992).

The high antioxidant activity of crude methanolic extract of *Anogeissus leiocarpus* is not surprising, because over the last two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole or their identified ingredients have antioxidant properties with substantial protective effects on human carcinogenesis, diabetes, asthma, atherosclerosis, and other degenerative diseases that have etiology and pathophysiology in reactive oxygen specie (Tsao *et al*., 2004; Atawodi *et al*., 2005; Valko *et al*., 2007). It has been suggested that this antioxidant property of plant products are mainly mediated by their content of phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Hollman and Arts, 2005; Manach *et al*., 2004; Lee *et al*., 2004; Vaya and Aviram, 2001).

The strong antioxidant activity of methanol *in vivo* is of enormous clinical significance, since natural antioxidants that protect against oxidative stress, can play vital roles in the prevention of diseases that have etiology and pathophysiology in reactive oxygen species For instance, *Anogeissus leiocarpus* has been reported to be used in different cultures as a recipe in the treatment of diabetes (Amusa *et al,* 2010), malaria **(**Vonthron-Sénécheau *et al.,* 2003), bacterial diseases (Adeleye *et al*., 2005), mycobacterial infections (Mann *et al*., 2009), fungal problems (Varaprasad *et al.,* 2009), and asthmatic attack *(*Sonibare and Gbile, 2008) . Thus, the observed antioxidant activity may in part, explain the mechanism by which this plant brings about some of these therapeutic effects.

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