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Research Article

EVALUATION OF ANTILIPID PEROXIDATION AND HYPOLIPIDEMIC POTENTIALS OF *AZADIRACHTA INDICA* LEAF AQUEOUS EXTRACT IN PARACETAMOL-INDUCED HEPATOTOXICITY IN WISTAR RATS

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ABSTRACT

Azadirachta indica, used widely in India, Nigeria and other parts of the world has been discovered to inhibit paracetamol-induced lipid peroxidation. Twenty four Wistar rats, placed into four groups of six rats each, were used. A (control) was given only normal saline; B was given paracetamol (800mg/kg body weight) to induced hepatotoxicity; C and D were given paracetamol (800mg/kg body weight) and *A. indica* aqueous leaf extract (400mg and 1000mg/kg body weight respectively). The animals were weighed before and after the experiment. The liver enzymes: AST, ALT and (ALP), serum and liver homogenate levels of MDA, and serum levels of triglycerides, cholesterol, HDL-c, LDL-c, were estimated. There was significant increase in the weights of A and D rats, decrease in B. ALT, AST, and ALP were significantly increased (P<0.05) in B compared to A and significantly decreased in C and D compared to B. The serum and homogenate levels of MDA were significantly increased (P< 0.05) in B compared to A, and significantly decreased in C and D compared to B. Triglycerides, total cholesterol and LDL-c were significantly increased (P<0.05) while HDL-c was decreased in group B. There were significant decreases (P<0.05) in triglycerides, total cholesterol, and LDL-c while HDL-c levels were increased in C and D, compared to B. These findings show that increases in lipid peroxidation, hyperlipidemia and hepatotoxicity found in paracetamol treated rats may have been effectively neutralized on treatment with the extract, suggesting that the extract may exhibits antilipid peroxidation and hypolipidemic potentials. It could also be hepatoprotective.

INTRODUCTION

Plants have long been recognized as synthetic media capable of making diversity of organic molecules that have complex structures and variety of physical, chemical and biological properties (Mayunga, 1996). These phytochemicals have the promise of ever remaining an inexhaustible source of medicines for the relief of man's many diseases and pains. In view of this, the leaf aqueous extract of *Azadirachta indica* (*A. indica*), an Indomalaysian plant has been demonstrated to offer protection against paracetamol-induced liver necrosis (Bhanwra, 2000). *A. indica* extract is composed of a complex mixture of molecules, including normal hydrocarbons, phenolic compounds, terpenoids, alkaloids, and glycosides (Sarawaneeyaruk, 2015; Hossain et al., 2013). The chemical constituents found in the leaves of *A. indica* are nimbin, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-benzoylgedunin, 17-hydroxy azadiradione and nimbiol (Kokate, 2010 and Hossain, 2013). Paracetamol has been reported to be hepatotoxic since 1966 (Thomson, 1966 and Davidson, 1999). The commonest enzymes regarded as indicators of liver damage are aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatases (ALP). The damage to hepatocellular cells results in increase in these enzymes activities (Nnodim, 2012). The elevated levels of serum liver enzymes AST, and ALT, indicative of liver damage, were found to be significantly reduced on administration of leaf aqueous extract of *A. indica* (Kausik, 2002). Hepatocellular activity of leaf aqeous extract of *A. indica* has been demonstrated by the finding that it offers protection against paracetamol-induced liver necrosis in rats. It has also been discovered that fresh juice of tender leaves of *A. indica* inhibited paracetamol-induced lipid peroxidation resulting to reduced production of MDA (Yanpallewar, 2003). MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic (Ayala, 2014; Esterbauer, 1990). Lipid peroxidation is a process by which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) (Ayala, 2014). It is a chain reaction initiated by the

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hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA). Lipid peroxidation concept recognizes the physiological effectiveness of the antioxidant defences in keeping both oxidative stress and cellular damage at a minimum level in physiological conditions (Boveris, 2008). These adverse effects may manifest significant alterations in the levels of biomolecules such as enzymes and metabolic products, normal functioning and histomorphology of the organs (Yakubu, 2009; Ashafa, 2012). Lipid peroxidation is considered as the main molecular mechanism involved in the oxidative damage to cell structures and in the toxicity process that lead to cell death. The toxicity of lipid peroxidation products in mammals generally involves neurotoxicity, hepatotoxicity, nephrotoxicity (Boveris, 2008), inflammation (Farooqui, 2011) and nutritional diseases (Repetto, 2010b).

The consequences of the peroxidative breakdown of membrane lipids have been considered in relation to both the subcellular and tissue aspects of liver injury. A more critical role is played by peroxidative reactions in the pathogenesis of acute liver necrosis induced by several pro-oxidant compounds as indicated by the protective effects against hepatocyte damage exerted by antioxidants (Poli, 1987). Reports indicate that changes in biochemical indices have been recorded in *A. indica* leaf extract fed rats; chemically induced carcinogenesis with accompanying high levels of lipid peroxidation in rats could be effectively reduced with *A. indca* leaf extract (Arivazhagan,, 200a). A five-day pre-treatment with this extract decreased the formation of lipid peroxidation and enhanced the level of antioxidants and detoxifying enzymes in the stomach, liver and circulation (Arivazhagan, 2000a). Any oxidative insult to a cell induces lipid peroxidation of cell membrane lipids. The lipid peroxidation products cause widespread damage of macromolecules. The peroxidation of lipid components of cells by reactive oxygen species (ROS) generates toxic species like lipid peroxides, lipid hydroperoxides and aldehyde breakdown products (Sherlock, 2000a). Oxidative stress, induced by paracetamol, is associated with increased formation of ROS that modifies phospholipids and protein, leading to peroxidation and oxidation of thiol groups (Malavi, 2004). The assaults by ROS lead to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins. In addition to these damages, abnormalities in myocyte function due to increased oxidative stress are considered to be associated with the effects of ROS on subcellular organelles (Kaneko, 1989).

Increased ROS production lowers cellular antioxidant levels and enhances the oxidative stress and, by extension, lipid peroxidation in many tissues, especially the liver (hepatotoxicity) (Hye-Lin, 2010). Hepatocellular activity of aqeous leaf extract of *A. indica* has been demonstrated by the finding that it offers protection against paracetamol-induced liver necrosis in rats (Bhanwra, 2000). Malondialdehyde (MDA) is the final product of lipid peroxidation (Marnett, 1999), and it can cross-link with amino group of proteins to form intra and inter molecular cross-links thereby inactivating several membrane bound enzymes (Kikhugawa, 1984), MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats (Marian Valko, 2007), Malondialdehyde is one of the most popular and reliable markers that determine oxidative stress in clinical situations (Giera, 2012), and is widely used to assess lipid peroxidation and oxidative stress. Since alteration in serum lipid profiles are likely to increase the risk of coronary heart diseases (Massing, 2001), a reduction in serum lipids particularly LDL and VLDL fractions and triglycerides levels should be considered beneficial in long term prognosis (Chattopadhyay, 2005). Thus, *A. indica* leaf extract may be helpful in controlling the development of hypercholesterolemia and artherosclerosis in view of its hypolipidemic action (Chattopadhyay, 1995), hence the need to evaluate its antilipid peroxidation and hypolidemic effects.

MATERIALS AND METHODS

Plant Materials

Procurement: Fresh matured leaves of *A. indica* were collected from a local neem tree at Ihiala, Anambra State, Nigeria, and identified at the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. A voucher specimen was deposited at the herbarium for further references.

***Extract preparation*:** The leaves were thoroughly washed and dried in carbonated moisture extraction drying oven (Grand instruments, Cambridge, England) at 45^0C - 50^0C for 3 hours. Grinding was done using Thomas contact mills (Py Unicam, Cambridge, England), The powder was sieved through 1mm sieve and 200g and soaked in 1000mls of water and allowed to stand for 48hours. The extract was filtered and the filtrate dried using hot air oven (Arrant instrument, Cambridge, England) at 45^0C - 50^0C. The residue yield was 52g and appropriate concentrations made for the experimental design using distilled water (*Nunomura,* 2006).

Paracetamol

A commercially available brand of paracetamol tablets (Emzor) were purchased from a registered pharmaceutical shop at Ihiala, Anambra State, Nigeria. The tablets were dissolved in distilled water (w/v) according to the required concentration for the administration on the Wistar rats on the basis of body weight.

Experimental Animals

Wistar rats weighing 150 - 250g were procured from the Animal House of College of Medicine and Health Science, Imo State University, Owerri. They were maintained under controlled conditions of light (12/24 hours) and temperature. The animals were fed with standard pellet diet (product of Pfizer, Nigeria Ltd) and allowed free access to water *ad libitum* throughout the period of the experiment (Chattopadhyay, 2005).

Experimental Design

Twenty four wistar rats were used in this study and randomly divided into four groups of six animals each as shown in Table 1. The leaf extract, paracetamol and normal were administered with the aid of a feeding canula.

Sample collection

After 28 days of treatment, all the animals were weighed and sacrificed via euthanasia using chloroform after a fasting of 16h following the last administration of food, aqueous leaf extract of *A. indica*, normal saline, paracetamol and water as

appropriate for each group of rats. Blood was collected by cardiac puncture, allowed to clot and then centrifuged at 10,000 revolutions per minute for 5 minutes using Wisperfuge model 1384 (Tamson, Holland). Serum was separated for various biochemical analysis and stored at -20° prior to use. The livers were dissected from all the animals, cleared of blood using normal saline and immediately transferred into blood ice-cold container of normal saline.

Table 1. Experimental design showing the groups of Wistar rats and the treatments they were given

Group	Treatment given
Group A (Control)	Received only normal saline (0.9% NaCl w/v) 5ml/kg body weight.
Group B	Received only paracetamol (800mg/kg body weight) once daily.
Group C	Received paracetamol (800mg/kg body weight) and *A. indica* leaves aqueous extract (500mg/kg bodyweight) once daily.
Group D	Received paracetamol (800mg/kg body weight) and *A. indica* leaves aqueus extract (1000mg/Kg bodyweight) once daily.

Acute Toxicity Testing

The acute toxicity of *A. indica* aqueous leaf extract was done using 30 mice divided into 5 groups of 6 mice each. Each group received graded doses (200 - 1000mg/kg body weight) of the extract and the animals observed for toxic effects after 48 hours of treatment. The toxicological effect was observed in terms of mortality expressed as LD_{50}. The number of animals that died during the period was noted. The LD_{50} of the extract was estimated from the graph of percentage (%) mortality, converted to probity, against log - dose of the extract, probit 5 being 50% (Litch Field. 1956).

Laboratory methods and procedures/biochemical analysis

The determination of AST and ALT was carried out using the Reitman and Frankel method (Reitman, 1957) while ALT was measured by King and King method (King, 1954). Malondialydehyde (MDA) was determined by the method of Albro *et. al.* (Albro, 1986) Triglyceride (TG) was determined using the Trinder method. Total cholesterol (TC) and high density lipoprotein cholesterol (HDL-c) were estimated by the method of Roeschlau *et al*. (Roeschlau, 1974).

LDL-c was estimated using the Friedewald's relationship: {LDL – c = Total cholesterol – (HDL + triglycerides/5)} (Friedwalds, 1972).

Statistical Analysis of Data

All values were expressed as mean ± SD and then subjected to analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) version 17.0 (SPSS Inc., Chicago Illinois). Statistical significance was considered at $\rho < 0.05$.

RESULTS

In Table 3, it was observed that there was a significant increase ($P<0.05$) in the liver homogenate level of MDA in group B (24.1±0.22) rats when compared with group A (13.3±0.44), while groups C (13.6±0.22) and D (12.7±0.21) did not show significant difference from group A. Group C and D showed significant decrease when compared with group B. This table also shows that the plasma MDA level in group B (7.79± 0.15) is significantly increased compared with group A (3.51± 0.16) while the plasma MDA levels in groups C (4.87± 0.13) and D (3.97± 0.56) indicate significantly decrease when compared with group B. Group C was significantly different while group D was not significantly different compared with group A.

Table 2. Mean weight changes (g) in Wistar rats treated with paracetamol

Groups	Initial weight (g)	Final weight (g)	Change in weight (g)
A	167±8.4	208 ±8.3	41± 6.2*
B	184 ± 3.7	175 ± 4.4	- 9±3.1*
C	171 ±5.8	177±5.6	6 ±4.2
D	194 ±5.8	218 ±5.1	24 ±4.1*

Key
*Significantly different ($p<0.05$) from initial weight.
Table 2 shows that there was a significant increase ($p<0.05$) in the body weight of rats in groups A and D, decrease in group B and no significant change ($p<0.05$) in the weights of Wistar rats in group C.

Table 3. Plasma and liver homogenate MDA levels of different groups of paracetamol treated Wistar rats treated with *A. indica*: aqueous leaf extracts and control

Groups	MDA	
	Homogenat (nmol/ml)	Serum (nmol/ml)
A	13.3 ± 0.44	3.51 ± 0.16
B	24.1 ± 0.22*	7.79 ± 0.152*
C	13.6 ± 0.22***	4.87 ± 0.13*
D	12.7 ± 0.21***	3.97 ± 0.56***

Key
*Significantly different ($P<0.05$) from group A
** Significantly different ($P<0.5$) from group A and B
*** Significantly different ($P<0.5$) from group B

Table 4. Plasma levels of hepatic marker enzymes of various groups of paracetamol treated Wistar rats with *A. indica* aqueous leaf extract, and control

Groups	AST (μ/I)	ALT (μ/I)	ALP (μ/I)
	80.4 ± 2.33	62.8 ± 3.25	80.4 ± 2.33
B	106.2± 2.56*	100 ± 4.1*	89.2 ± 3.12*
C	91.6 ± 3.44**	98.6 ±3.93*	86.2 ± 2.71
D	69.2 ± 2.48***	65.6 ± 2.8***	80.1 ± 3. 12***

Key
*significantly different ($p<0.05$) from control group A
**Significantly different ($p<0.5$) from group A and B
***Significantly different ($p<0.05$) from group B.

Table 4 shows that there was significantly area ($p<0.05$) in the plasma levels of the liver enzymes AST, ALT, and ALP in group B (106.2 ±2.56,100±4.1, 89.2±3.12 respectively) compared to those of group A. (60.6±3.98, $62.8^{\pm}$3.25, $80.4^{\pm}$2.33 respectively). The plasma level of AST in group C Wistar rats was significantly increased ($p<0.5$) compared with group A but significantly decreased when compared with group B. the plasma ALT level in group C was significantly increased when compared with group A but no significant difference from group B. There was no significantly variation in plasma ALP level of group C from those of groups A and B rats. This table also shows that there was no significant difference in the plasma levels of AST, ALT and ALP in group D (69.2 ±2.48, 65.6±2.8, 80.1± 3.12 respectively) compared with group A. There was, however, significant decrease in plasma level of AST, ALT and ALP in group A compared with group B. It was evident from the table that there were significant difference in the plasma levels of ALP between group C (86.2 ±2.71) and D. In Table 5, the plasma levels of total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol

(LDL-c) of group B (202±4.43, 147.6±2.25, 152.1±33 respectively) show significant increase when compared with group A. (108±2.56,90, 90.4±3.5, 59.1±2.52 respectively) Wistar rats. Group B (20.±3.14) show significant decrease in plasma value of HDL-c compare with group A. (30.8±2.71).

Table 5. Lipid profile of different groups of paracetamol treated Wistar rats treated with *A. indica* aqueous leaf extract and control

Groups	Total chlesterol (mg/dl)	Triglycerides (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)
A	108±2.56	90.4±3.5	30.8±2.71	59.1±2.52
B	202±4.43*	147.6±2.25*	20.4±3.14*	152.1±2.33*
C	168.8±2.32**	126 ±3.41**	24.8±2.48*	118.8±3.02**
D	116±3.1***	98.6±2.33***	27.8±2.04***	63.5±2.24***

Key
*Significantly different ($p<0.5$) from group A
**Significantly different ($p<0.5$) from groups A and B
***Significantly different ($p<0.5$) from group B

Group C show significant increase ($p<0.5$) in plasma TC, TG, and LDL-c levels when compared with group A. and a significant decrease when compared with group B. There was no significant difference between the plasma levels of C and LDL-c of groups D and A. Group C plasma level of HDL-c indicates significant decrease from A. but not significantly different from group B. Group D (116. ±3.1, 98.6±2.88, 63.5±2.24 respective) shows significant decrease ($p<0.5$) in these parameters, TC, TG and LDL-c with reference to group B and no significant difference when compared with group A. Group D (27.8±2.04) plasma level of HDL-c shows significant increase when compared with group B but no significant difference from group A. This table also shows that the variation between the plasma level of TC, TG, and LDL-c of groups C and D were significant while their plasma levels of HDL-c were not significant ($p<0.05$) compared to group D.

DISCUSSION

In this study, it appears that the overall effect of various treatments of the different groups, used in this study, was summarized in their respective weight changes. The significant weight loss in paracetamol treated Wistar rats may be attributed to the negative biochemical effect engendered by the induced paracetamol toxicity, while the no significant weight change observed in the ones treated with both paracetamol and *A. indica* aqueous leaf extract reflects the inhibitory effect of *A. indica* aqueous leaf on paracetamol-induced hepatotoxicity. The observed increase in the hepatic marker enzymes, AST, ALT and ALP, in paracetamol treated Wistar rats agrees with its already documented hepatotoxic effect. And the no significant change demonstrated by these enzymes in the Wistar rats treated with both paracetamol and *A. indica* extract is also consistent with documented reports on hepatoprotective activity of *A. indica* (Chattopadhyay, 1995). The elevated levels of these hepatic enzymes in paracetamol treated Wistar rats (group B) are also well correlated with the alteration in the hepatocellular integrity. The significant increase in liver enzymes and MDA, which are indicators of increased lipid peroxidation and its resultant hepatotoxicity, in paracetamol intoxicated Wistar rats, as observed in this study supports the concept that paracetamol over-dose could result to hepatotoxicity (Nnodim, 2012) and consequent cell death. The mild but significant effect on ALP may mean that though paracetamol intoxication causes hepatocellular damage (Hazai, 2002), cholestasis may not be primarily involved. The significant increase in total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-c) and decrease in high density lipoprotein (HDL-c) in paracetamol treated Wistar rats (groups B) compared with their respective levels in the control (group A) in this study are consistent with established lipid profile in hepatocellular damage. But for the rats treated with paracetamol and varied doses of *A. indica* aqueous leaf extract (groups C and D), the significant decrease in TC, TG, LDL-c, and increase in HDL-c compared with those of the paracetamol treated group indicate the hypolipidaemic effect which might have resulted from antilipidaemic role of *A. indica* aqueous leaf extract. This finding is consistent with documented results that, the effect of *A. indica* leaf extract on serum and liver lipid parameters viz. cholesterol, total lipids, phospholipids and triglycerides, indicates that it exhibits significant hypolipidemic activity in rats (Chattopadhyay, 1995). The lipid peroxidation product (MDA) level was increased in paracetamol treated Wistar rats. This finding is consistent with the previous works reported by (Yanpallewar, 2003). The increase in MDA implies increased lipid peroxidation as a result of hepatotoxicity which could have been induced by paracetamol, as shown in table 4: The no significant change in MDA of groups C and D compared with control (group A) animals and the significant decrease when compared with group B animal, may mean that *A. indica* aqueous leaf extract can offer protection against paracetamol-induced free radical injury and thus prove to be a good antidote to oxidative stress and its resultant cell death (hepatotoxicity). This may imply that the hepatotoxicity induced by paracetamol was almost completely obliterated by the concomitant administration of *A. indica* aqueous leaf extract. Consequently, the observed variations in the elevated or reduced levels of biochemical parameters evaluated in this work, with regards to varied doses of *A. indica* extract used, can suggest that its effect in controlling oxidative stress and hepatotoxicity may be dose-dependent.

Conclusion

Based on the findings in this study, it could be inferred that *A. indica* aqueous leaf extract may have antilipid peroxidation, hypolidemic and hepatoprotective effects on paracetamol-treated Wistar rats. This could be achieved by either directly scavenging the reactive oxygen metabolites or enhancing the levels of antioxidant molecules which, in turn, could inhibit lipid peroxidation resulting to hypolipidemia. Having observed the invaluable role *A. indica* play in attenuating or obliterating oxidative stress conditions including hepatocellular damage by its antilipid peroxidation and hypolidemic effects, it is recommended that further studies should be carried out to elucidate the molecular basis of both the biochemical an d pharmacologic involvement of this plant extract in achieving this effect.

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