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Full Length Research Paper

Roles of *Glyphaea brevis* (*Spreng*) extract in cadmium induced hepatocellular damage and oxidative stress in rabbit

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This study investigated the roles of the ethanolic extract of the leaves of *Glyphaea brevis* (Spreng) in cadmium induced hepatocellular damage and oxidative stress in rabbit. Sixteen (16) rabbits weighing between 2.0 and 2.2 kg were divided into four groups of four animals and treated orally as follows: Group I; 2 ml of normal saline per day and Group II; cadmium chloride (1.25 mg/kg body weight per day). Animals in group III and IV were pretreated with ethanolic extract of *Glyphaea brevis* (300 mg/kg body weight per day) and Vitamin E (300mg/kg body weight per day) respectively. One hour later cadmium chloride solution (1.25mg/kg body weight per day) was administered to animals in group III and IV. The animals were treated for 10 days and sacrificed on day 11. Hepatic marker enzymes; (Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Gamma Glutammyl Transferase (GGT)) activities were determined in the plasma and liver homogenates. Plasma albumin, total protein, uric acid and bilirubin concentrations were also estimated according to standard procedures. The results revealed that the activities of all hepatic marker enzymes in the plasma were elevated in cadmium only treated group when compared with control, positive control (Vitamin E+ cadmium group) and extract treated group (*Glyphaea brevis* + Cadmium). It also caused statistically significant (P<0.05) decrease in plasma albumin, protein and uric acid concentrations. Also, there was significant (P<0.05) increase in bilirubin concentration in cadmium only treated group compared with other experimental groups. Administration of ethanolic extract of *Glyphaea brevis* one hour prior to cadmium exposure reduced the toxic effects of cadmium on the liver of rabbits. Based on the results of this study, the roles of *Glyphaea brevis* extract may include protection of the liver and as antioxidant against cadmium-mediated oxidative stress.

Key words: *Glyphaea brevis*, Cadmium, Rabbit, Oxidative stress, Hepatotoxicity

INTRODUCTION

Pharmaceutical industries have come to consider traditional medicines as a source of identification of bio-active agents that can be used in the preparation of synthetic medicines. Extract from medicinal plants are sold in the partially purified or crude form for the treatment and prevention of all kinds of diseases. Most of these herbal products lack scientific backing for the various efficacies claimed. Plants extracts are used for disease conditions such as mental disorders, diabetes, sickle cell anemia, malaria, tuberculosis and a host of other diseases in traditional medicine throughout the world (Odugbemi, 2008).

Glyphaea brevis is a spreading shrub, climber or small tree up to 8m high. It is very common in undergrowth of closed forest, secondary jungle and on river-banks, lowlands to sub-mountain and wide spread in tropical Africa (Burkill, 1985). It is widely distributed in Africa

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and South America. It is valued there as vegetable (Okafor, 1990) and various therapeutic uses such as treatment of hepatitis and poisoning have been reported (Terasima and Ichikawa, 2003). *Glyphaea brevis* (Spreng) which is popularly called Aloanyasi (Ibo) or Atori (Yoruba). It has been reported to have multiple physiological and pharmacological activities. It is used in the treatment of sleeping sickness and as aphrodisiac, as an antibacterial in the treatment of eye infection and in gum cleaning. It is also reported to be effective in the treatment of impotency (Vasilea, 1969). It has carminative effects and is used as an anticonvulsant, especially in children, where it is either used singly or in combination with other herbs (Ijomah, 1996; Ogbonnia et al., 2003).

Therapeutic activities of various medicinal plants are sometimes related to their antioxidant properties (Agbor *et al.,* 2007). Therefore antioxidant activity could be accountable for the medicinal properties of *Glypheaa brevis* through a contribution to redox homeostasis. Heavy metals are known to constitute serious threats to human health (Wennerberg, 1994). Cadmium being one of these heavy metals is a wide spread environmental pollutant, it is characterized by its toxicity in various organs (Gunnarsson *et al.,* 2003). Cadmium induces serious peroxidation in membrane structures (Stohs *et al.,* 2000). The molecular mechanisms responsible for the toxic effects of cadmium include interference with antioxidant enzymes (Hussain *et al*., 1987), alterations in the structure of thiol proteins (Li *et al.,* 1991) and alteration in DNA structure (Yu and Chen, 2004). Acute cadmium exposure primarily results in accumulation of the metal in the liver, causing acute hepatotoxicity (Liu *et al.,* 1996). Metals and metalloids affect almost every organ of the body. One such metal is cadmium, which is of concern because of its increasing prevalence as an environmental contaminant (Jarup*,* 1998). Prolonged exposure to cadmium results in injury to liver, lung, kidney, and testes (Manca *et al*., 1991).

Hepatotoxic effects of cadmium are evidenced by increased plasma levels of alanine aminotransferase (ALT), aspartate aminotraerases (AST) and alkaline phosphatase (Dudley *et al*., 1982). Histological evaluation of liver damage induced by cadmium exposure reveals that acute toxicity provokes parenchymal cell necrosis, infiltration of inflammatory cells (Tzirogiannins, *et al.,* 2004), hepatocellular swelling, sinusoidal congestion, pyknosis and karyrrhexis (Dudley *et al*., 1982).These cellular changes may result in both apoptosis and necrosis. Moreover, release of apoptogenic proteins is likely related to calcium induced alteration of mitochondrial homeostasis which is also preceded by production of reactive oxygen species (Lemarie *et al.,* 2004). Over production of reactive oxygen species (ROS) has been considered as the primary mechanism for cadmium toxicity (Rikans and Yamano, 2000). Beneficial effects were documented for treatment with antioxidant against cadmium-induced oxidative stress in rat livers (Tandon *et al.,* 2003).

The current study was designed to investigate the roles of ethanolic extract of the leaf of *Glyphaea brevis* in cadmium- induced hepatotoxicity in rabbits.

MATERIALS AND METHODS

Materials

Collection and identification of plant

Fresh leaves of *Glypaea brevis* (Spreng) were collected from Ede road, Ile-Ife, Nigeria. The plant was identified and authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife by Mr. Ibhanesebor, G.A where the specimen copy was deposited.

Reagents and chemicals

All the reagents used in the study were of analytical grade. They were obtained from British Drug House Limited (BDH) London, Sigma Fine Chemicals Limited, Upsalla, Sweden, Fluka Chemical Company Plc. Germany. Reagent kits to assay for the enzymes were obtained from Randox Laboratories Ltd, United Kingdom. All solutions, buffers and reagents were prepared with glass-distilled water.

Experimental Animals

Sixteen healthy female rabbits with an average weight range of 1.8 kg to 2.2 kg were purchased from the Teaching and Research Farm, Obafemi Awolowo University, Ile-Ife. The animals were acclimatized for 4 weeks in the animal house where they had free access to clean water and standard pellets (Guinea Feeds, Benin City, Nigeria).

Methods

Preparation of ethanolic extract of *Glyphaea brevis*

The leaves of *Glyphaea brevis* were air dried under laboratory condition for 28 days. The dried leaves were milled with mellon milling machine and sieved. Five hundred (500 g) of the powdered leaf was subjected to soxhlet extraction with 4 litres of petroleum spirit for four hours to remove the fat and chlorophyll, followed by further extraction using 4 litres of 70% (v/v) ethanol for six hours. The ethanolic extract was concentrated on Edward Vacuum Evaporator at 35^0C. The extract was subjected to phytochemical screening to confirm the major constituents present.

Phytochemical Screening

Phytochemical screening was carried out using the method of Edeoga *et al*. (2005).

Test for tannins

The extract (0.5g) was boiled in 20 ml of water in a test tube and then filtered. Two drops of 0.1% (w/v) ferric chloride reagent were added to 5 ml of the filtrate and observed for brownish, green or blue-black colouration. Appearance of brownish, green indicated the presence of tannins.

Test for saponins

The filtrate (10ml) was mixed with 5 ml of distilled water and shake vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil shaken vigorously, and then observed for the formation of emulsion.

Test for flavonoids

The extract (5 mg) was heated in 10 ml of ethylacetate over a steam bath for 3 min. The mixture was filtered and 4 ml of filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed, indicating a positive test for flavonoid.

Test for steroids

Acetic anhydride (0.2 ml) was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The colour was expected to change from violet to blue or green if steroids were present.

Test for terpenoids (Salkowski test)

The extract 0.5 g was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration at the interface was formed to show positive result for the presence of terpenoids.

Test for cardiac glycosides (Keller Killani test)

Ethanolic extract 0.5 g treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxy sugar, characteristics of cardenolides. A violet ring appears below the brown ring, while in the acetic acid layer, a greenish ring formed gradually throughout thin layer.

Treatment of Experimental Animals

Sixteen New Zealand female rabbits weighing 1.8 - 2.2 kg were isolated for weeks. The rabbits were caged individually and cages were tagged for easy identification during treatment. They were randomly divided into four groups of four animals (Groups I to IV) and treated as follows:
Group I (Normal saline) – Animals in this group received 2 ml/kg body weight, per day of normal saline.
Group II (Cadmium) – Animals in this group received cadmium chloride solution only (1.25 mg/kg body weight) per day. Group III (Extract treated) – Animals in group 3 were pretreated with ethanolic extract (300mg/kg body weight per day) and one hour later, cadmium chloride (1.25 mg/kg body weight per day) was administered to each of the animals.
Group IV (Positive control) – Each animal in this group was given vitamin E (300mg/kg body weight per day) one hour before the administration of cadmium chloride (1.25 mg/kg body weight per day)

Biochemical Studies

Plasma and liver homogenate were prepared according to standard procedures as described by Babalola and Areola (2010).

Digestion of liver

One gram (1 g) of liver was homogenized in 5 ml of (Conc. $HN0_3$: HCl 1:4 v/v), the homogenate was poured into a test tube, covered with cotton wool and left on the bench overnight to digest the tissue. The sample was heated at 100^0C in water bath for 20 minutes and allowed to cool before the addition of 1.0 ml of hydrogen peroxide (H_2O_2). The sample was then diluted to a final volume of 25 ml with distilled water and stored in a 30 ml polyethylene bottle for later analysis by AAS (Atomic Absorption Spectroscopy).

Estimation of Biochemical parameters

Total protein concentration in the plasma and liver homogenate were estimated using Biuret reaction method (Gornal *et al.,* 1949). In alkaline medium, Cu^{2+} reacts with peptide bonds of protein to give a blue violet coloured complex which absorbs maximally at 540 nm. The absorbance of the coloured complex is proportional to the concentration of the protein.

The determination of albumin procedure used in this study was a modification of that of Pinnel and Northan (1978). In the presence of a solubilizing agent, BCP binds to albumin at pH 4.9. The amount of albumin-BCP complex is directly proportional to the albumin concentration. The complex absorbs at 600nm.

The plasma total bilirubin was estimated as described by Jendrassik and Grof (1938) .Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin by the reaction with diazotized sulphanilic acid.

Uric acid in the plasma was estimated as described by Fossati et al., (1980). Uric acid is converted by uricase to allantoin and hydrogen peroxide, which under catalytic influence of peroxidase, oxidizes 3,5-Dichloro-2-hydroxybezene sulfonic acid and 4-aminophenazone to form a red violet quinoneimine compound which absorb maximally at 520nm.

Activity of the hepatic marker enzymes

The plasma and liver homogenates ALT and AST activities were estimated as described by Reitman and Frankel (1957) using the Randox kit.
The procedure was based on the formation of Pyruvate from L-alanine and α-oxaloglutarate by the action of the enzyme which was monitored at 546nm after the formation of Pyruvate hydrazone with 2, 4-dinitrophenylhydrazine.

Table 1. Results of the phytochemical screening.

Test	Result
Cardiac glycosides	+
Flavonoids	+
Saponin	+
Steroids	_
Terpenoids	+
Tannins	+

+ Positive; - Negative

Table 2. Effects of cadmium and *Glyphaea brevis* extract on the activities of hepatic marker enzymes in the plasma.

Group	ALT	AST	GGT
Control	16.40 ± 4.91	38.00 ± 6.00	1.74 ± 0.58
G.brevis + Cadmium	17.15 ± 0.50	55.50 ± 4.50	2.27± 0.12
Vit. E + Cadmium	9.57 ± 1.92	43.00 ± 7.00	1.74± 0.58
Cadmium only	40.71 ± 4.08	92.00 ± 8.00	31.27 ± 1.16

Values are in U/L and expressed as mean ± SEM (standard error of mean)

Plasma GGT (gamma glutamyl transferase) activity was estimated as described by Szasz (1969). The substrate L- δ - glutamyl-3-carboxy-4-nitroanilide, in the presence glycylglycine is converted by δ-GT in the sample to 5-amino-2-nitrobenzoate which was measured at 405nm.

RESULTS

The yield of ethanolic extract from 500 g of dried leaf of *Glyphaea brevis* was 72.5 g representing 14.5% yield of the starting material. Phytochemical screening of the extract showed the presence of cardiac glycosides, flavonoids, tannins, terpenoids and saponins.

Cadmium concentration analyses by Atomic Absorption Spectroscopy showed that the cadmium concentration in animals treated with cadmium only was 0.14µg/g while only 0.01µg/g was found in animals treated with cadmium and extract as well as cadmium and vitamin E. There was no cadmium in the liver of the animals in control group.

Statistical analyses

All data obtained from various experiment were subjected to statistical analysis using graph pad. Values are expressed as mean± standard error of mean (SEM). The results were analyzed using student's t-test and analysis of variance (ANOVA) and values of $P<0.05$ were taken to be statistically significant.

DISCUSSION

The yield of 14.5% obtained was higher than 8.5% reported by Ogbonnia *et al*., (2003), the difference may be due to the method of extraction or the specie of the plant. Phytochemical screening of the extract confirmed the presence of Cardiac glycosides, Flavonoids, Saponins, Terpenoids and Tannins as shown in Table 1.

The presence of cadmium in the liver of the animals confirmed previous findings that acute cadmium exposure primarily results in accumulation of the metal in liver causing hepatotoxicity (Babalola and Areola, 2010). Lower concentration of cadmium in the liver of the animals pretreated with vitamin E appears to establish the fact that vitamin E was able to reduce the cadmium burden due to it protective activity as an antioxidant. Similar effect observed in the liver of the animals pretreated with extract maybe due to inherent antioxidant properties of the plant extract (Dakam *et al*., 2008). Medicinal plants owe their therapeutic properties to the presence of different phytochemicals in their leaves, stem bark, roots and fruits (Sofowora, 2008). The presence of phenolic compounds (Flavonoids and tannins) in Glyphaea brevis extract maybe responsible for this protection.

Liver is the target organ for cadmium toxicity; Cadmium administration to experimental animals leads to morphological and functional hepatic changes (Tzirogiannis, *et al*., 2004). The results of the study showed significant increase in the activities of plasma Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Gamma glutamyl transferase (GGT) in cadmium treated group (Table 2) but there was no statistically significant increase in the activity of liver enzymes in the blood of the animals pretreated with the extract when compared with the control group. This increase may be due to lipid

Table 3. Effects of cadmium and *Glyphaea brevis* extract on the activities of hepatic marker enzymes in the liver.

Groups	ALT	AST
Control	39.71 ± 1.75	61.00 ± 1.00
G.brevis + Cadmium	45.12 ± 2.50	59.00 ± 1.00
Vit. E + Cadmium	44.37 ± 4.25	58.00 ± 1.00
Cadmium only	21.40 ± 1.75	33.50 ± 10.50

Values are in U/L and expressed as mean ± SEM (standard error of mean)

Table 4. Effects of cadmium and *Glyphaea brevis* extract on the concentration of metabolites in the plasma.

Groups	Total Protein	Albumin	Bilirubin	Uric acid
Control	27.13 ± 3.84	15.18 ± 2.34	30.96 ± 1.12	0.44 ± 0.19
G.brevis + Cadmium	18.18 ± 0.37	13.59 ± 0.39	35.93 ± 0.73	0.49± 0.02
Vit. E + Cadmium	17.76 ± 1.90	13.28 ± 0.72	35.34 ± 1.13	0.42± 0.10
Cadmium only	2.42	2.69 ± 0.13	43.11 ± 3.03	1.15 ± 0.13

Values are in mg/dl and expressed as mean ± SEM (standard error of mean)

peroxidation of bio-membranes which causes leakage of cellular components (Matsuo, *et al.,* 1989). Exposure of hepatocytes to cadmium stimulates cellular production of H_2O_2 which affects permeability barrier of plasma membranes (Koizumi, *et al.,* 1994).

Significant decrease in the concentration of plasma protein and albumin in cadmium treated group showed that Plasma total protein and albumin concentration decreased from 27.13 mg/dl and 15.59 mg/dl in control group to 10.35 mg/dl and 2.69 mg/dl in cadmium treated group respectively (Table 3). This significant reduction in the concentration of these metabolites is an indication of severe liver injury; concentration of total protein and albumin in plasma are commonly used to evaluate liver function. Significant reduction in the concentration of these metabolites is an indication of severe liver injury because liver is the principal organ responsible for these proteins but albumin synthesis is also sensitive to amino acid supply and thus nutrition state plays important roles in albumin concentration (Herper, 1961).

Also, there was an increase in the concentration of plasma total bilirubin (Table 4) from 30.96 ± 1.12 mg/dl in control group to 43.11 ± 3.03 mg/dl in cadmium only group. Bilirubin is a break down product of haem containing proteins. The liver is responsible for clearing the blood of bilirubin through conjugation process. Increased total bilirubin (conjugated and unconjugated) in the plasma may be an indication of liver injury. Low concentration of albumin can also affect the rate of bilirubin conjugation; as observed in this study (Nyblom *et al.,* 2006).

In addition, significant decreased in the concentration of plasma uric acid was also observed in cadmium treated group when compared with the other groups. Plasma uric acid significantly decreased from 0.21 ± 0.16 in control group to 0.01 ± 0.008mg/l in cadmium treated group but there was no statistically significant decrease in plasma uric acid of the animals pretreated with the extract when compared with the control group. Low level of uric acid in the blood can be associated with some kinds of liver diseases (American association for clinical chemistry, 2001). Uric acid, the metabolic end product of purine metabolism, has been shown to be a selective antioxidant, capable of reacting with free radicals and hypochlorous acid (Hasugawa and Kuroda, 1989).

Conclusion

In conclusion, the results suggested that the roles of *Glyphaea brevis* extract may include antihepatotoxic and antioxidant, since the extract of *Glyphaea brevis* was able to minimize the toxic effect of cadmium on the liver. These findings further justified the use of various parts of the plant in traditional medicine for the treatment and management of diseases.

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