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Acute Toxicity, Polyphenolic Composition and Liver Protective Capabilities of the Methanol Leaf Extract of *Alangium salvifolium* (LIN. F) WANG (Alangiaceae)

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Abstract

Medicinal plants over the years have been explored for the prevention, management and treatment of diverse disease conditions. Different part of the plant *Alangium salvifolium* has been used in folklore medicine for the management and treatment of ulcer, microbial infection, free radical implicated disorders among others. The study aims to investigate the oral toxicity profile and the extent of liver protection from carbon tetrachloride-induced toxicity. The Lorke's oral acute toxicity, polyphenolic composition as well as the protective effect of the methanol extract on the liver enzyme markers (aspartate transaminase (AST), alanine phosphatase (ALP), alanine transferase (ALT), glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT) and malondialdehyde (MDA)) in carbon tetrachloride (CCl_4) intoxicated rats model, was evaluated using standard procedures. The results showed a marked reduction in the serum activities of AST, ALP, ALT, and MDA with an attendant increase in the levels of GSH, GPx and CAT when compared to the induction control animal group. The 221.38 mgGAE/g and 131.56 mgQE/g for the total phenolic and total flavonoid contents respectively could be responsible for the 62.70% free radical scavenging activity as well as the hepatoprotective effect of the plant extract reported. There was no mortality after 24 hr oral administration of the extract at the highest investigated concentration of 4 g/kg p.o and a further 14 days of observation. In conclusion, the present findings suggest that *A. salvifolium* can offer some degree of hepatic protection from chemicals-induced toxicity in experimental animals.

Keywords: *Alangium salvifolium*, Carbon tetrachloride, Liver markers

Introduction

Medicinal plants have since time immemorial been explored for their medicinal properties in managing and treating the diverse disease conditions plaguing mankind [1]. Globally, scientific researchers have established that medicinal plants are potent source of therapeutic drugs capable of ameliorating natural and chemical induced toxicity [2]. In Nigeria specifically, medicinal herbs are widely explored in treating different disease conditions as a result of their affordability, easy accessibility and effectiveness [3].

The toxic effects of chemicals on several internal organs and cellular components such as liver, kidneys, brain, hematopoiesis, lipids as well as proteins and nucleic acids have been over the years

been a cause of public health concern [4]. Carbon tetrachloride (CCl_4) is an organic solvent used diversely in many pharmaceutical industries used as an hepatotoxic agent for the study of the pathogenesis of liver injury [5]. CCl_4 acts by interacting with membrane lipids thus bringing about peroxidation whose overall end product of is the production of malondialdehyde (MDA) or 4-hydroxynonenal (HNE) which is a highly reactive aldehydes capable of forming adducts with protein and DNA [6,7].

The excess production of these reactive oxygen species (ROS) tends to overwhelms the body endogenous antioxidant defense system bringing about oxidative stress implicayted in the pathogenesis of many disease conditions [8]. The body endogenous antioxidant enzymes notably superoxide dismutate (SOD), catalase (CAT), glutathione peroxidase (GPx) and as well the non-antioxidant enzyme glutathione (GSH) [9]. CCl_4 have been reported to bring about an increased lipid synthesis accompanied by a decrease in the transport of lipids out of the hepatocyte [10,11]. This imbalance between the synthesis and degradation of lipids have been postulated to be the direct cause of steatosis associated with CCl_4 - induced hepatotoxicity [12].

Alangium salvifolium (AS) native to tropical Australia, Madagascar, western Africa, eastern Asia (China, Malaysia, Indonesia, India, and Philippines) and New Caledonia is a small deciduous tree growing up to 3 to 10 m height, with more or less spine scent branches and pale brown bark [13]. The plant is commonly known as "*sage-leave alangium*" or "*purple sage*". Locally in Nigeria it is known as "*Salwa*" in Hausa", "*Ewe koro*" in Yoruba and "*Nchuanwu isi oji*" in Igbo. Ethnomedicinally, *AS* is a versatile medicinal plant showing potent antidiabetic, anticancer, diuretic, anti-inflammatory, antimicrobial, laxative, astringent, emollient, anthelmintic, hepatoprotective and antiepileptic activities among others [14].

Despite the therapeutic uses of silymarin and colchicine known to protect the liver from CCl_4 - induced damage, they are known to produce troubling side effects like severe throat pain, bone marrow suppression, rhabdomyolysis, bloating among others hence the need to search for efficient and readily available hepatoprotective agent from natural source that could either be used directly or serve as lead compounds for the treatment of liver disease is worthy of scientific investigations.

Figure 1: *Alangium salvifolium* plant in its natural habitat

Materials and Methods

Collection and Identification

The plant was identified through ethnobotanical approach. Healthy and mature plant leaves were

collected openly from Agbani in Nkanu East local government area of Enugu state. The plant materials were identified and authenticated at the Department of Pharmacognosy and Ethnomedicine, Faculty of Pharmacy, Madonna University Elele, Rivers Nigeria. The plant materials were thoroughly rinsed with running tap water to remove the dirt and specks of dust before been shade dried at room temperature for two weeks. The dried leaves were reduced to fine powder using an electric mill machine, sieved and stored in an airtight glass container until ready for extraction.

Extract preparation

Powdered plant material (1.00 kg) was extracted with 5.0 L of absolute methanol by maceration at room temperature for 72 hours. The extract was concentrated to dryness using a rotary evaporator at 40^{o}C under reduced pressure. The concentrated extract was air dried, weighed, stored in an air-tight container and refrigerated at 4^{o}C until ready for use

Antioxidant analysis

Total phenol content (TPC)

TPC in the extract was determined according to the Folin-Ciocalteu procedure [15] with slight modifications. The extract solution (0.5 mL) with a concentration of 1000 µg/mL was added to 4.5 mL of deionized distilled water and 0.5 mL of Folin Ciocalteu's reagent. The solution was maintained at room temperature for 5 minutes followed by the addition of 5 mL of 7 % sodium carbonate and 2 mL of deionized distilled water. The thoroughly mixed samples were incubated for 90 minutes at 23°C. The absorbance was measured by spectrophotometer at 750 nm. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. Gallic acid was used as positive control. The standard curve was prepared using gallic acid at different concentrations

Total flavonoid content (TFC)

The total flavonoid content in the plant extracts was determined by the Aluminum Chloride method as described [16]. A 0.5 mL portion of the extract with a concentration of 1000 µg/mL was mixed with 1.5 mL of methanol and then, 0.1 mL of 10 % aluminum chloride was added, followed by 0.1 mL of potassium acetate and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. The results were expressed as milligrams Quercetin equivalents (QE) per gram of extract (mg QE/g dry extract). Quercetin was used as positive control and the standard curve was prepared using quercetin at different concentrations.

DPPH free radical scavenging activity

The ability of methanol extract of *E. marginata* to scavenge 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was estimated as previously described [17] with slight modifications. A 3 mL portion of the extract solution with 1000 µg/mL) were mixed with 1 mL of a 0.1 mM methanolic solution of DPPH.

The absorbance was measured by a spectrophotometer at 517 nm at 30 minutes intervals against a blank (pure ethanol). The percentage of radical scavenging activity was calculated using the formula:

$$\text{DPPH radical inhibition (\%)} = [1 - (\text{A test}/\ \text{A conrol})]\ \text{X}\ 100$$

Where A **c**ontro**l** is the absorbance of the control and A test is the absorbance of the extract. Ascorbic acid was used as a reference standard with the same concentrations.

Animal's procurement and treatment

Fifty (50) adult Wistar rats of both sexes weighing 150 - 180 g were purchased from a local breeder in Nsukka Enugu State. They were transported and housed in the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Madonna University Elele Rivers State, Nigeria. The animals were housed in standard cages and fed with rodent feed and water *ad libitum.* The animals were acclimatized to laboratory conditions for 14 days before the experiments. After obtaining ethical approval (MUN/FP/24/008), the study was conducted in accordance with the principle governing the handling procedures of experimental animals as laid down by the Madonna University, Nigeria ethical committee on animal use and care. Efforts were made to minimize the number of animals used and their sufferings by strictly following the ethical guidelines for investigations of experimental pain in conscious animals as described.

Acute toxicity study

Twenty five (25) Wistar albino rats were fasted for 12 h (overnight) prior to the experiment. The animals were divided into five groups of five animals per group. Prior to the start of the experiment, the body weights of the animals were recorded to calculate the proper treatment dosage. The animals in groups A – D were administered with single dose of *A. salvifolium* methanol extract orally at doses of 0.5, 1, 2, and 4 g/kg body weight respectively. The fifth group (group E) served as the control and was administered 1.00 mL of distilled water. All animals were observed for 24 h for mortality. In an event of death, the lethal dose of 50% of the animals tested (LD_{50}) would be determined and if no death occurs changes in general behaviors, physical condition, waste, mortality and changes in body weight will be made for a further 14 days after extract administration [18].

CCl_4 induced toxicity

The protocol as described by El-Hashash *et al*. [19] was adopted with slight modifications. Twenty five (25) albino rats were divided into five groups, each group consisting of five animals grouped as shown in the intervention Table 1

Table 1: Intervention groups of rats based on treatment plan

Group	Group Code	Description
A	Negative Control (NC)	Received 0.5 mL of distilled water daily for a period of 30 days
B	CCl_4 –induced (CCl_4 – I)	Received CCl_4 intraperitoneal injection (i.p.) at a dose of 2 mL /kg body weight once every week on the 1st, 8th, 15th and 30th day of treatment
C	Standard Control (SC)	Received CCl_4 intraperitoneal injection (i.p.) at a dose of 2 mL /kg body weight once every week on the 1st, 8th, 15th and 30th day of treatment in addition to silymarin (p.o.) at a dose of 16 mg/kg body weight for 30 days
D	Extract Control (EC)	Received CCl_4 intraperitoneal injection (i.p.) at a dose of 2 mL /kg body weight once every week on the 1st, 8th, 15th and 30th day of treatment in addition to *A. salvifolium* extract (p.o.) at a dose of 400 mg/kg body weight for 30 days
E	Standard Extract Control (SEC)	Received CCl_4 intraperitoneal injection (i.p.) at a dose of 2 mL /kg body weight once every week on the 1st, 8th, 15th and 30th day of treatment in addition to silymarin (p.o.) at a dose of 16 mg/kg body weight and *A. salvifolium* extract (p.o.) at a dose of 400 mg/kg body weight for 30 days

After 7 days of experiment, all the animals were decapitated by overnight fasting and using chloroform anesthesia. The blood was collected in heparinized and non-heparinized tubes. Serum was separated by centrifugation at 4000 rpm for 20 min and stored in a refrigerator at 20^{o}C until analysis. The liver tissue was collected and perfused with normal saline to remove blood and used for the preparation of tissue homogenate. Liver function test namely alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) as well as antioxidant enzyme activity namely catalase (CAT), malondialdehyde **(**MDA), glutathione (GSH), glutathione peroxidase (GPx) were performed.

Biochemical analysis

Determination of ALP, ALT and AST

The activity of ALP was determined according to the procedure outlined by King and Armstrong (1934) while that of ALT and AST were determined according to the procedures outlined by Reitman and Frankel [20].

Antioxidant enzyme assay

Catalase (CAT)

The activity of CAT was measured as described by Aebi [21]. The reaction principle is based on CAT reacting with excess hydrogen peroxide (H_2O_2) with the excess H_2O_2 reacting with 3,5 - dichloro - 2 - hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore capable of absorbing light whose colour intensity is inversely proportional to the absorbance measured at 240 nm. The enzymatic activity of CAT was expressed in units/mg cellular protein.

Malondialdehyde (MDA)

The activity of MDA was measured as described by Uchiyama and Mihara [22]. The reaction is based

on the principle of the determination of the concentration of thiobarbituric acid (TBA) as a marker for MDA used to determine the extent of lipid peroxidation in the liver. 0.5 mL of tricholoacetic acid (TCA) was mixed with the liver homogenate. The mixture was centrifuged for 10 min at 3500 rpm. 1.0 mL of the supernatant and 1.0 mL of TBA were added and heated on a water bath for 10 min. The solution was then placed in a cold bath and its absorbance taken at 535 nm. The result of MDA is expressed as μmol/g.

Glutathione (GSH)

The activity of GSH was measured as described by Beutler *et al* [23]. 200 μL of the liver homogenate was placed in an Eppendorf tube and mixed with 300 μL of deionized water and 300 μL of precipitating solution (containing sodium chloride, EDTA, and glacial meta phosphoric acid in concentrations of 30, 0.2 and 1.67 g). The resulting mixture was then incubated for 5 min and immediately centrifuged for a further 10 min. 200 μL of the supernatant was placed in a cuvette with 800 μL phosphate solution and 100 μL DNTB. The absorbance was measured at 412 nm with reference to a blank solution.

Glutathione peroxidase activity (GPx)

The activity of GSH was measured as described by Paglia and Valentine [24]. GPx catalyzes the oxidation of glutathione. GSH - Px activity was measured spectrophotometrically at 340 nm The result of GPx is expressed as unit/mg-protein.

Statistical Analysis

The results of all parameters were stated as mean with standard deviation (Mean ± SD). Using one - way analysis of variance (ANOVA), the data were statistically analyzed. Tuckey's test was applied among different treatment groups to make a comparison and to identify the statistical difference. $P < 0.05$ was considered as statistically significant.

Results and Discussion

Percentage yield of crude extracts

Approximately, 41.70 g (4.17%) viscous mass was obtained from 1.0 kg powdered leaf extract of *A. salvifolium* after 72 h of cold maceration in absolute methanol

TPC, TFC and DPPH assay

Polyphenols are diverse secondary plant metabolites reported to possess antioxidant activity. The result of the TPC, TFC, and DPPH assay of *AS* extract are represented in Table 2.

Table 2: TPC, TFC and DPPH assay of *Alangium salvifolium* extract

Parameter	Concentrations
TPC (mg GAE/g of dry extract)	221.38
TFC (mg QE/g of dry extract)	131.56
DPPH radical scavenging effects %	62.70

Phenolic are potent free radical scavengers as a result of them acting as metal chelators [25]. Reported researches have established that phenolic compounds exhibit biological activities such as

antioxidant, anti-diabetic, hepatoprotective, anti-inflammatory, antimicrobial, anticancer among others [26].

The reported free radical scavenging activity in the DPPH model employed for this study could be attributed to the phenolic and flavonoid content as suggested by Zhang and Lin [27] who stated that plants with high phenolic and flavonoid content exhibits potent antioxidant activity thou other polyphenolics like anthocyanidins, carotenoids, steroids among others could partly contribute to the reported antioxidant activity.

Acute toxicity test

The effect of *A. salvifolium* extract on the body weights after 14 days is shown in Figure 2.

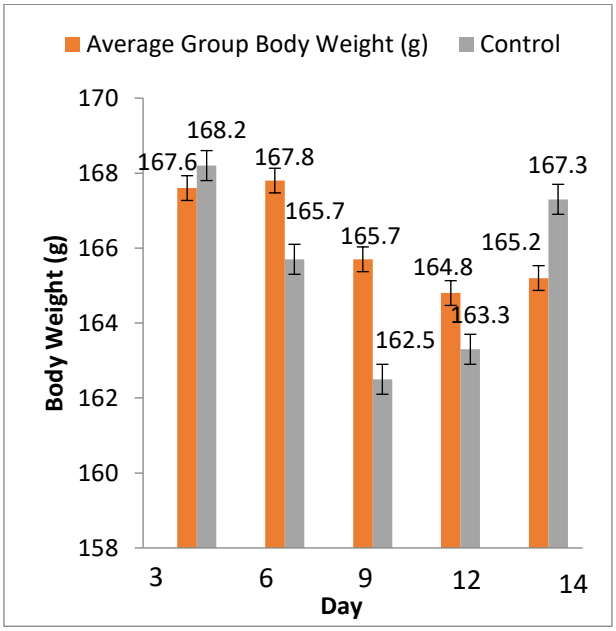

Figure 2: Body weights of rats in 14 days. Data are expressed as Mean ± SEM and analyzed using Student's t-test (n = 5 per group)

Oral administration of the *AS* extract at the highest dose of 4.0 g/kg resulted in no mortality or signs of acute toxicity in RAT, as observed for an initial period of 24 hrs and for an extended observation period of 14 days. As shown in Figure 2, the body weights of the treated group fluctuates slightly with that of the control but thou not statistically significant when compared to that of the control group. The result showed that the LD_{50} dose of *AS* is greater than 4 g/kg body weight and hence could be deduced not to have acute toxicity according to the Guidelines for Preclinical and Clinical Trials of Traditional Medicine and Herbal Medicine by the Vietnam Ministry of Health [28].

Liver Enzymes Markers (ALP, ALT and AST)

The liver is susceptible plays vital role in the neutralization and excretion of xenobiotics. The mechanism of CCl_4 - induced hepatic injury is believed to involve the biotransformation of CCl_4 to trichloromethyl (CCl_3), and trichloromethyl peroxyl radicals. These radicals are very reactive causing lipid peroxidation and decreased activities of the antioxidant defense system in the liver tissues [29]. Elevated levels of these liver enzymes in the plasma could be attributed to the likely leakage of these enzymes from liver tissue into the plasma occasioned by LPO of cell membranes which had been reported to be the cause of loss of membrane fluidity, changes in membrane potential and an increase in membrane permeability. The result of the 30 days administration of *AS* extract on the investigated liver marker enzymes is presented in Table 3 below:

Table 3. Effect of *AS* extract on liver enzymes in CCl_4 - induced hepatotoxity in Wistar rats

Parameter	Group				
	A	B	C	D	E
ALP (U/L)	88.55±6.1	145.26±8.2[a]	104.82±5.1[a,b]	102.2 ±6.2[a,b]	92.73±7.9[a,b]
ALT (U/L)	33.12±.4	63.25 ±4.4[a]	54.34 ±4.1[a,b]	50.20 ±5.3[a,b]	38.75±1.9[b]
AST (U/L)	42.10±3.4	110.33±4.8[a]	72.22 ±4.4[a,b]	68.19±7.7[a,b]	50.57±3.7[b]

Data are expressed as Mean ±SD for the five animals in each group. [a] $p<0.05$ compared with negative control (Group A). [b] $p<0.05$ compared with CCl_4 –induced group control (Group B).

The result presented in Table 3 confirms the ability of CCl_4 to induce liver damage as there was statistically significant elevated levels of the hepatic markers (ALP, ALT, AST) in the CCl_4 induced group (Group B) which did not receive any form of intervention when compared to that of the normal control group (Group A). When used alone, silymarin (Group C) and the *AS* extract (Group D) produced a significant decrease in the hepatic marker enzymes when compared to the CCl_4 induced group (Group B) hence confirming their hepatoprotective capabilities.

However, when both silymarin and the *AS* extract (Group E) where administered concurrently, there was a remarkably decline in the hepatic marker enzymes and almost brought it back to normal (Group A levels). This confirms their individual hepatoprotective activity as well as their synergistic hepatoprotective activity. This result therefore strongly suggests that the hepatoprotective effect of *AS* extract may be related to its ability to mitigate against LPO which in turn stabilizes the integrity of the hepatic tissue membranes thus preventing the leakage of these liver enzymes and as well capable of stimulating hepatic cell regeneration and preserve the structural integrity of hepatic membrane [30,31].

Antioxidant enzyme assay (CAT, MDA, GSH and GPx)

The study revealed that CCl_4 bring about a significant ($p<0.05$) increase in MDA levels signifying an increase in lipid peroxidation with a corresponding decrease in CAT, GSH and GPx levels ($p<0.05$). These results are presented in Table 4.

Table 4: Effect of *AS* extract on antioxidant enzymes in CCl_4 - induced hepatotoxity in Wistar rats

Parameter	Group				
	A	B	C	D	E
MDA	4.12±0.77	27.11±1.35[a]	10.56±1.35[a,b]	11.43±1.23[a,b]	7.41±1.11[b]
CAT	33.44±2.22	11.88±0.66[a]	27.77±1.11[a,b]	20.34±1.22[a,b]	29.77±1.22[b]
GSH	21.66±1.21	12.55±1.03[a]	19.44±1.33[a,b]	17.44±1.44	29.88±0.66[a,b]
GPx	31.10±21.12	18.55±1.22[a]	25.47±1.11[a,b]	22.16±1.33[a]	27.11±1.46[b]

Data are expressed as Mean ±SD (n = 5). [a] $p<0.05$ compared with the negative control (Group A). [b] $p<0.05$ compared with CCl_4 - induced group control (Group B).

The MDA trend as reported in Table 3 could be attributed to free radical generation from the peroxidation of polyunsaturated fatty acids [32]. The increase in MDA level in this study confirmed the pro-oxidant and hepatotoxic nature of CCl_4, manifested likely due to the failure of antioxidant defense mechanisms in the hepatocytes to prevent the formation of excessive free radicals [33]. This agrees to the observed results as Group B lacking in any form of antioxidative intervention reported the highest MDA level unlike in Groups C, D and E where silymarin, the extract and the co-administration of silymarin and the extract respectively prevent the formation of excessive free radicals which bring about a decrease in MDA levels (Al-Dosari, 2010).

The observed decreased in the CAT, GSH and GPx levels in group B when compared to groups C, D and E could be attributed to the generated free radicals overwhelming the free radical defense systems thou increased in concentration in groups C, D and E respectively after 30 days supplementation with silymarin and the extract. Thou the standard drug silymarin (Group C) had a better antioxidant activity than the *AS* extract (Group D), it exhibited a lower activity than group E when the co-administration of the drug and the extract was used. This suggests the positive synergistic antioxidant activity of the standard silymarin and the extract.

In effect, the liver membrane damage caused by CCl_3OO^- following the intraperitoneal injection of CCl_4 was largely protected by the *AS* extract at the investigated concentration in this study. This may be due to the ability of the extract to stabilizes the integrity of the hepatic tissue membranes thus preventing the leakage of these liver enzymes in addition to its ability to mitigate against LPO of cell membranes which had been reported to be the cause of loss of membrane fluidity, changes in membrane potential and an increase in membrane permeability [18].

Conclusion

The present study offers scientific backing on the regenerative role of *Alangium salvifolium* extract induced by CCl_4 toxicity in rats. Our finding suggested that the plant extract restored the liver functioning in exposed rats by ameliorating the level of serum liver enzymes accompanied by its antioxidant activity. The phenolic and flavonoid compounds present in the extract offer hepato-protection which have been attributed to their antioxidant and anti-inflammatory potential. More studies are needed to actually ascertain the underlying mechanism of its hepatoprotective effect

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