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Hepatoprotective and antioxidant effects of *Acanthospermum hispidum* (DC) leaves on carbon tetrachloride-induced acute liver damage in rat

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ABSTRACT

The leaves of *Acanthospermum hispidum* (DC) are used traditionally by Togolese to treat various hepatic disorders. The aim of the present study was to evaluate the hepatoprotective effect and antioxidant activities of the hydroethanolic extract of *A. hispidum* on carbon tetrachloride (CCl₄)-induced liver damage in Wistar rats. The extract was also studied for its *in vitro* antioxidant activity using ferric thiocyanate (FTC) and 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) methods. The hydroethanolic extract of *A. hispidum* administered orally at 250 and 500 mg/kg showed a significant hepatoprotective effect ($P < 0.001$) by reducing levels of alanine amino transaminase (ALT), aspartate amino transaminase (AST) and alkaline phosphatase (ALP). The extract exhibited significant hepatoprotective and antioxidant activities. These data suggest that the hydroethanolic extracts of *A. hispidum* can prevent hepatic injuries from CCl₄-induced hepatotoxicity in rats and this is likely mediated through its antioxidant activities.

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Keywords: *Hepatoprotective, Antioxidant, Acanthospermum hispidum, CCl₄.*

INTRODUCTION

The liver regulates many important metabolic functions. Hepatic injury is associated with distortion of these metabolic functions (Wolf, 2000). Additionally, it is the key organ of metabolism and excretion. Then it is continuously and variedly exposed to xenobiotics because of its strategic placement in the body. The toxins absorbed from the intestinal tract gain access first to the liver resulting in a variety of liver ailments. Thus liver diseases remain one of the serious health problems (Didem et al., 2007). Modern medicines have little to offer for

alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for their treatment of liver disorders. But there are not much drugs available for the treatment of liver disorders (Chatterjee, 2000). Therefore, many folk remedies from plant origin are evaluated for its possible antioxidant and hepatoprotective effects against different chemical-induced liver damage in experimental animals. CCl₄-induced hepatotoxicity model is frequently used for the investigation of hepatoprotective effects of drugs and plant extracts. The changes associated with CCl₄-induced liver

damage are similar to that of acute viral hepatitis (Suja et al., 2002). *A. hispidum* is herbal medicinal plant which is distributed over large areas in Togo. The ethanolic extract of the sheets and the flowers of *A. hispidum* presents an inhibiting activity on the development of *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhi* and *Clostridium histolyticum* (Fleischer et al., 2003); antiplasmodial activity (Sanon et al., 2003) and antifungal (Hoffman et al., 2004) properties has been demonstrated. The plant is commonly used in Togolese folk medicine to treat various diseases. *A. hispidum* is used in the treatment of leprosy. The whole decoction of the plant is given like purgative and antidote. It is used in the treatment of the evils of belly, the wounds and the migraines. It is used in the treatment of the hepatic affections. The possible scientific hepatoprotective activity of *A. hispidum* leaves has not been reported. Therefore, this study aimed at evaluating the hepatoprotective and antioxidant effects of *A. hispidum* leaves by using the CCl₄-induced acute liver injury model in rats.

MATERIALS AND METHODS

Plants materials

Leaves of *A. hispidum* were collected in July 2007 in Agoè, located at 15 km in the North of Lomé (Togo). The samples of the plant was identified at the Laboratory of Botany and vegetable Ecology of the Faculty of Science, University of Lomé, Togo and the voucher specimen was deposited (TOGO12717) in the herbarium. The leaves were washed, dried under shade and powdered coarsely before extraction.

Animals

Male and female Wistar rats (150–200 g) were used. The animals were bred at the Laboratory of Physiology/ Pharmacology of the Faculty of Sciences, University of Lomé. They were housed under standard environmental conditions. They had a 12 hours light/dark cycle. The animals had free

access to food and water. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals.

Preparation of the plant extract

The powder of *A. hispidum* (247 g) was macerated in a mixture ethanol distilled water (1:1) during 72 hours under manual agitation intermittently. The filtrate was evaporated under vacuum with 45 °C by a rotary evaporator (the rotavapor Buchi R210). Yield was 23.48%.

Phytochemical screening

Preliminary phytochemical composition of the hydroethanolic extract was studied using the following tests (Itoria et al., 2011): alkaloids with Mayer's and Dragandroff's test, flavonoids with Shinoda and Zinc hydrochloride reduction test, tannin with Ferric chloride and Vanillin hydrochloride test and saponins with the ability to produce suds.

Determination of total phenols

Gallic acid was used to make the calibration curve [0.05; 0.15; 0.2; 0.25 mg/mL]. 500 µl of extract or gallic acid were mixed with 0.2 mL Folin-Ciocalteu reagent 2 mL of H₂O, and 1 mL of 15% Na₂CO₃ and the mixture was measured at 765 nm after 15min at room temperature. The mean of three readings was used and the total phenolic content was expressed in milligram of gallic acid equivalents/g extract (Didem et al., 2007).

Determination of total flavonoids

Flavonoids were expressed as quercetin equivalent. Quercetin was used to make the calibration curve [1; 5; 12.5; 25; 50; 75; 100 µg/mL]. The standard solutions or extracts (500 µl) were mixed with 1.5 mL methanol, 0.1 mL 10% aluminum chloride (w/v), 0.1 mL of 1 mol/L sodium acetate and 2.8 mL distiller water. The volume of 10% aluminum chloride was substituted by the same volume of

distilled water in blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The mean of three readings was used and the total flavonoid content was expressed in milligram of quercetin equivalents/g extract (Didem et al., 2007).

CCl₄-induced hepatotoxicity

We induced the hepatotoxicity with CCl₄ according to the method of Suja et al. (2004). The animals were divided into 7 groups of 7 animals each. Group I served as normal control and received distilled water (0.5 mL/100g of body weight (bw), per os) daily for 3 days and received corn oil (2.5 mL/kg per os) on day 3; 60 min after the administration of distilled water. Group II served as CCl₄ control and received distilled water (0.5 mL/100g, per os) daily for 3 days and received CCl₄ : corn oil (1:1), (2.5 mL/kg bw, per os) on day 3; 60 min after the administration of distilled water. Groups III-IV were treated with of *A. hispidum* at doses of 250, and 500 mg/kg per os, respectively, for 3 days and received CCl₄ : corn oil (1:1), (2.5 mL/kg bw., per os) on days 3; 60 min after administration of extract. Group VI was treated with the reference drug silymarin at dose of 100 mg/kg per os for 3 days and received CCl₄ : corn oil (1:1), (2.5 mL/kg bw., per os) on day 3; 60 min after administration of silymarin.

Forty eight (48) hours after the administration of CCl₄ the animals were anaesthetized with ether, then blood was taken on the level of the retro-orbital sine. This blood was centrifuged and in the serum the biochemical parameters such as: Alanine aminotransferase (ALT), the aspartate aminotransferase (AST), phosphatase alkaline (PAL) were proportioned.

Assessment of antioxidant activities of extracts

FeCl₂-ascorbic acid stimulated lipid peroxidation in liver homogenate

The antilipid peroxidant effect of *A. hispidum* was studied *in vitro*, following the modified method of Jin et al. (2005). Briefly,

2 g of rat liver tissue was sliced and homogenised with 150mM KCl-Tris-HCl buffer (pH 7.2). The reaction mixture was composed of 0.35 mL liver homogenate, 0.1 mL Tris-HCl buffer (pH 7.2); 0.05 mL of FeCl₂ 4 mM; 0.05 mL of ascorbic acid 0.1 mM (AA) and 0.05 ml of various concentrations of *A. hispidum* extract. The mixture was incubated at 37 °C for 1 hour in capped tubes. Then 0.5 mL of HCl (1N), 0.2 mL of sodium dodecyl sulphate (SDS: 9.8%), 0.9 mL distilled water and 2 mL of thiobarbituric acid (TBA: 0.6%) were added to each tube and vigorously shaken. The tubes were placed in a boiling water bath at 100 °C for 30 min. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 25 min. The absorbance of the supernatant was measured at 532 nm.

Anti-Hemolysis activity of A. hispidum induced by 2,2,-azobis(2-amidinopropane hydrochloride)(AAPH)

The Anti-Hemolysis Activity of *A. hispidum* was studied *in vitro*, following the method of Qin et al. (2002), Yifan et al. (2009) and Nitya et al. (2012). Rats were anaesthetised with ether. Blood (5–7 ml/rat) obtained from the sine retro-orbital was collected into heparinized tubes. Erythrocytes were separated from plasma and the buffy coat, and were washed three times with 5 vol of phosphate-buffered saline (PBS), pH 7.4. During the last wash, the erythrocytes were centrifuged at 3000 g for 10 min to obtain a packed cell preparation. The packed erythrocytes were then suspended in 4 vol of PBS solution. The peroxidation of erythrocyte membranes was studied using a Azo compounds (AAPH) generate free radicals by their unimolecular thermal decomposition in the aqueous region. Two milliliters of the erythrocyte suspension were mixed with 2 ml of PBS solution containing varying amounts of *A. hispidum* (2.5–40 M). Two milliliters of 200 mM AAPH in PBS were then added to the mixture. The reaction mixture was shaken gently while being incubated at 37 °C for 3 hours. After incubation, the reaction mixture was diluted with 8 vol of PBS and was centrifuged at 3000g for 5 min. The absorbance (A) of the supernatant fraction at 540 nm was

recorded in a spectrophotometer (Beckman Instruments, Fullerton). The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [A_{\text{AAPH}} - A_{\text{extract}} / A_{\text{AAPH}}]$$

Where A_{extract} is the absorbance of the sample containing either the extract or ascorbic acid, and A_{AAPH} is the absorbance of the sample containing neither extract nor ascorbic acid was used as a positive control.

Five replicates were performed for each extract.

Statistical analysis

The results were analyzed using the software Systat 11.0. They are expressed as mean \pm SEM. One way analysis of variance (ANOVA) followed by Fischer's "Least Significant Difference" was used to compare the various groups. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Effects of *A. hispidum* extract on serum AST, ALT and PAL levels in CCl_4 -intoxicated rats

Administration of CCl_4 to rats caused significant ($p < 0.05$) increase in serum enzymes like AST, ALT, and PAL, compared to normal control rats. Treatment with *A. hispidum* (AH) caused significant ($p < 0.05$) reduction of these values, almost comparable

to the Silymarin (S) treated group (Figures.1-3).

Effects of *A. hispidum* on oxidative stress

Effects of *A. hispidum* on FeCl_2 -ascorbic acid stimulated lipid peroxidation

A. hispidum showed very potent inhibition of FeCl_2 -AA stimulated rat liver lipid peroxidation *in vitro*. There was a significant ($p < 0.05$) increase of malondialdehyde (MDA) in FeCl_2 -AA treated rat liver homogenate, compared to normal control without FeCl_2 -AA (Table 1).

Effects of *A. hispidum* on AAPH-induced hemolysis of erythrocytes

The influence of *A. hispidum* extract on *in vitro* erythrocyte hemolysis was examined by incubating rat erythrocytes in the presence of 200 mM AAPH as an initiator of oxidation. The *A. hispidum* extract provided a strong inhibitory effect against erythrocyte hemolysis. The extract inhibited *in vitro* AAPH-induced erythrocytes hemolysis in a dose-dependent manner (Table 2).

Phytochemical screening

Qualitative phytochemical tests revealed the presence of alkaloids, flavanoids, tannins and saponins (Table 3). Quantitative phytochemical studies showed that the extracts contained phenols and flavonoids (Table 4).

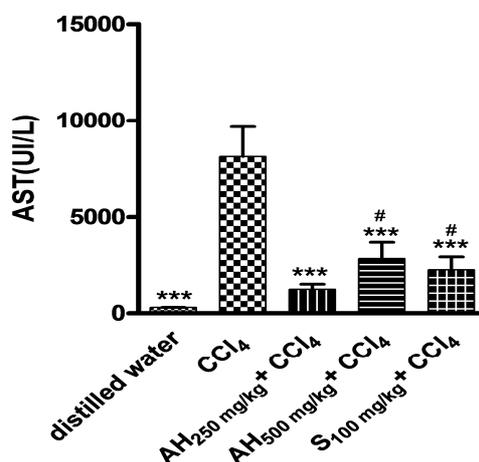


Figure 1: Effects of *A. hispidum* extract on serum AST levels in CCl_4 -intoxicated rats. The data are expressed as mean \pm S.E.M ($n = 7$). * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$ as compared with the CCl_4 control # $P < 0.05$ as compared with distilled water. The various groups were compared by ANOVA followed by Fischer' LSD.

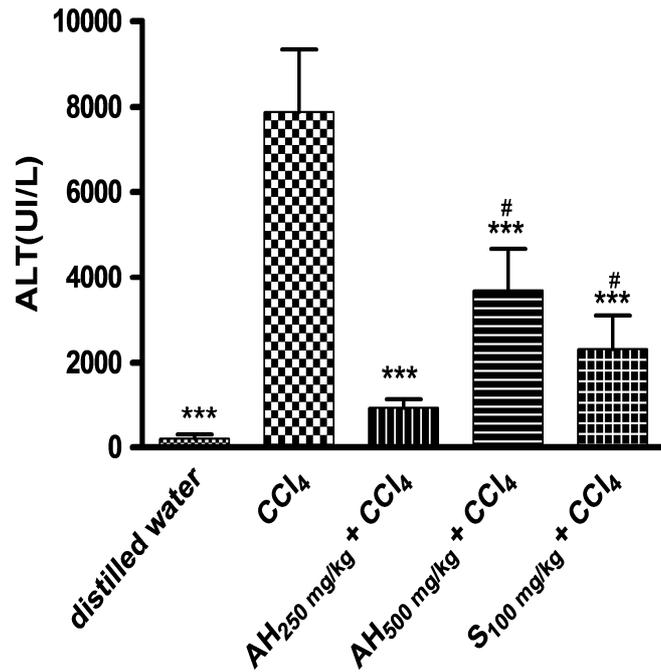


Figure 2: Effects of *A. hispidum* extracts on serum ALT levels in CCl₄-intoxicated rats. The data are expressed as mean ± S.E.M ($n = 7$). * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$ as compared with the CCl₄ control, # $P < 0,05$ as compared with distilled water. The various groups were compared by ANOVA followed by Fischer' LSD.

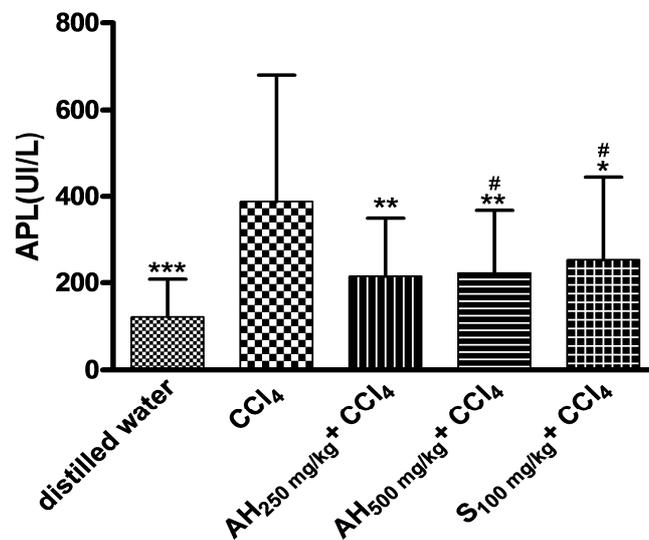


Figure 3: Effects of *A. hispidum* extract on serum PAL levels in CCl₄-intoxicated rats. The data are expressed as mean ± S.E.M ($n = 7$). * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$ as compared with the CCl₄ control, # $P < 0,05$ as compared with distilled water. The various groups were compared by ANOVA followed by Fischer' LSD.

Table 1: Inhibitory effect of *A. hispidum* extract on FeCl₂-ascorbic acid-induced lipid peroxidation in rat liver homogenate *in vitro*.

Treatment	Dose (mg/ml)	MDA (nM/g of liver)
Normal control		12.47 ± 2.10
FeCl ₂ -AA		78.73 ± 4.95
FeCl ₂ -AA + Quercetin	0.05	50.53 ± 0.70*
	0.10	38.31 ± 1.10**
	0.15	36.90 ± 6.10**
	0.20	30.79 ± 5.90**
	0.25	22.33 ± 5.50**
FeCl ₂ -AA + <i>A. hispidum</i>	0.05	59.93 ± 4.48*
	0.10	51.94 ± 2.00*
	0.15	32.20 ± 1.50**
	0.20	12.53 ± 3.70***
	0.25	35.49 ± 4.11**
	0.25	14.35 ± 4.48***

The data are expressed as mean ± S.E.M (n = 3). *P < 0.05, **P < 0.01, *** p < 0.001 compared to FeCl₂-AA group.

Table 2: Effects of *A. hispidum* on AAPH-induced hemolysis of erythrocytes. Data are expressed as mean ± SEM.

Treatment	Dose (mg/ml)	% of inhibition	IC ₅₀ (mg/ml)
Ascorbic Acid + AAPH	0.10	84 ± 0.004	0.06
	0.25	96 ± 0.003	
	0.50	97 ± 0.001	
	1.00	98 ± 0.002	
<i>A. hispidum</i> + AAPH	0.10	05 ± 0.044	0.43
	0.25	31 ± 0.036	
	0.50	56 ± 0.029	
	1.00	98 ± 0.010	

Table 3: Phytochemicals tests in hydroethanolic extract of *A. hispidum*.

Experiment	<i>A. hispidum</i> extract
Alkaloids	++
Flavonoids	++
Tannin	+++
Saponins	+++

+++ : very abundant ; ++ : Abundant.

Table 4: Total phenolic and total flavonoid contents of *A. hispidum*.

Extracts	Total phenolic content (mg GAE/ g extract)	Total flavonoid content (mg QE/g extract)
<i>A. hispidum</i>	155 ± 0.6	25.43 ± 0.4

GAE = gallic acid equivalent; QE = quercetin equivalent.

DISCUSSION

CCl₄-induced hepatotoxicity model is frequently used for the investigation of hepatoprotective effects of drugs and plant extracts. The changes associated with CCl₄-induced liver damage are similar to that of acute viral hepatitis (Suja et al., 2002). CCl₄ is biotransformed by cytochrome P-450 in the liver endoplasmic reticulum to the highly reactive trichloromethyl free radical (CCl₃[•]). This free radical in turn reacts with oxygen to form a trichloromethyl peroxy radical (CCl₃OO[•]). Both radicals are capable to binding proteins or lipids, or abstracting a hydrogen atom from an unsaturated lipid, thus, initiating lipid peroxidation, generate oxidative stress (Didem et al., 2007). This state involves the depletion of natural antioxidants such as the superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) (Suja et al., 2004). It results from the hepatolysis which increasing the rate of the serum enzymes such as ALT, AST and ALP (Shanmugasundaram et al., 2006). Transaminases and alkaline phosphatase are plasmatic enzymes of hepatic origin which make it possible to explore the state of liver (Suja et al., 2004). A significant rise in the serum concentration of these enzymes is a sign of hepatic intoxication. In this study, ALT, AST and ALP activities levels significantly increased in rats treated with CCl₄ and decreased in rats treated with CCl₄ and *A. hispidum* (250 mg/kg and 500 mg/kg). *A. hispidum* thus have hepatoprotectives effects. Clinical studies reported that reactive oxygen species are associated with many diseases, including hepatitis, liver injury (Lee et al., 2004). In order to clarify the mode of action of *A. hispidum in vitro*, lipid peroxidation experiments were carried out. Lipid peroxidation is accepted to be one of the main causes of carbon tetrachloride-induced liver injury, and is mediated by the production of free radical derivatives of carbon tetrachloride. Thus, antioxidant activity and/or the inhibition of free radicals generation is important in terms of protecting the liver from carbon tetrachloride-induced damage (Hye et

al., 2002). *In vitro* lipid peroxidation in a liver homogenate can proceed in a nonenzymatic way. The process is induced by ascorbate in the presence of Fe²⁺/Fe³⁺, and it has been reported that Fe²⁺ and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria (Yong et al., 2008). According to the results obtained, *A. hispidum* inhibited FeCl₂-ascorbic acid stimulated lipid peroxidation in liver homogenate (Table 2). *A. hispidum* was found to be effective in decreasing malondialdehyde (MDA) production in liver of rats treated with FeCl₂-ascorbic acid showing its antilipid peroxidation effects. AAPH is a water soluble radical generator that produces a range of free radicals at a constant rate without the use of enzymes or biotransformation (Nitya et al., 2012). Therefore, oxidative hemolysis of erythrocytes induced by AAPH and its protection by compounds act as a good model system to screen the test compounds for their antioxidant behavior (Arnab et al., 2008). According to the results obtained, *A. hispidum* extract also inhibited AAPH induced oxidative hemolysis of erythrocytes (Table 2). Phenolic antioxidants are reported to be able to quench oxygen-derived free radicals as well as substrate-derived free radicals, protect cell constituents against oxidative damage and, therefore, limit the risk of various diseases associated to oxidative stress (Scalbert et al., 2005). The results of the qualitative characterization indicate an abundance of flavonoids in each extract. These results are confirmed by the quantitative tests (Table 3). Fleischer et al. (2003) showed that *A. hispidum* contains polyphenols. There is a correlation between the quantity of flavonoids and the antioxidant capacity ($r^2 = 0.9846$ and $p < 0.001$). Flavonoids have an anti-hepatotoxic activity (Lahouel et al., 2004; Amani et al., 2006). This suggests that in fact, the flavonoids confer on extract the antioxidant capacity which is at the base of the hepatoprotectives effects (Didem et al., 2007). Plant antioxidants have been shown to protect erythrocytes against hemolysis induced by AAPH. Qin et al. (2002) reported

that treating rat erythrocytes with a cocoa extract containing flavonoid inhibits hemolysis induced by AAPH *in vitro*. Arnab et al. (2008) reported that treating erythrocytes with curcumin (flavonoid) inhibits AAPH-induced hemolysis, as evidenced by reduced release of hemoglobin. Similarly, Yifan et al. (2009) reported that treating erythrocytes with quercetin (flavonoid) inhibits AAPH-induced hemolysis. The investigators attributed the antihemolytic actions of these plant-derived compounds to their antioxidant property (Yifan et al., 2009). Increasing evidence suggests that flavonoids protect cell not only by buffering free radicals but also by altering cell membrane properties (Arora et al., 2000). Like *in vitro* lipid peroxidation inhibition induced by AAPH free radicals (R^{\bullet} and ROO^{\bullet}), *A. hispidum* compound inhibited *in vivo* lipid peroxidation induced by CCl_4 free radicals, CCl_3^{\bullet} and CCl_3OO^{\bullet} . The correlation between the quantity of flavonoids and the antioxidant capacity suggests that flavonoids attributed to the extract its antioxidant properties which were at the base of its hepatoprotectives effects.

Conclusion

A. hispidum leaves has hepatoprotectives effects that explains its use in traditional medicine. The active compounds of *A. hispidum* leaves, which are responsible for antioxidant and hepatoprotectives effects, have not been isolated in this study. Therefore, further studies should be conducted to isolate the active compounds that are responsible for the hepatoprotectives effects.

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