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EVALUATION OF HEPATOPROTECTIVE AND NEPHROPROTECTIVE ACTIVITIES OF ETHANOLIC EXTRACT OF *PHYLLANTHUS AMARUS* SCHUMACH. & THONN AGAINST CCL_4-INDUCED OXIDATIVE STRESS AND DAMAGE IN WISTAR RATS.

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ABSTRACT
Vegetable drugs are taken recurrently to improve or cure pathological processes, without any scientific knowledge of their pharmacodynamic activities. The aim of this study was to evaluate the effects in liver and kidney of *Phyllanthus amarus* used in virus hepatitis treatment. Carbon tetrachloride (CCL_4) is used to induce toxicity whose main target organs are liver and kidney (hepatotoxicity and nephrotoxicity). After poisoning (CCL_4), the animals are treated curatively with the extracts, according to the model of Fleurentin and Joyeux. The hepatic and renal parameters investigated are transaminases (ASAT, ALAT), alkaline phosphatase (PAL), bilirubin (free and conjugated), urea, total protein, creatinine. All data is processed using Microsoft Excel 2010 and was analyzed by One-Way Analysis of the variance (ANOVA) followed by Tukey's post-test for the comparison of the averages. The threshold of significance is 5%. Several doses (250 mg/kg, 500 mg/kg, 750 mg/kg) of the ethanolic extract of *P. amarus* were used to evaluate effective doses for liver and kidney. Biochemical analysis show a significant decrease in transaminases (ASAT, ALAT), alkaline phosphatase (PAL), bilirubin (free and conjugated) at 500 mg/kg. Concerning renal parameters, we notice a significant decrease in urea, creatinine at 750 mg/kg and a significant increase in total protein at the same dose. The ethanolic extract of *P. amarus* protect respectively liver and kidney against the oxidative stress of CCL_4 at 500 mg/kg and 750 mg/kg.

KEYWORDS: Hepatotoxicity, Nephrotoxicity, *Phyllanthus amarus*, Carbon tetrachloride.

INTRODUCTION
African populations use traditional medicines to meet their primary care needs health.[1] The Beninese flora contains about three thousand species[2], including *Phyllanthus amarus* Schumach. and Thonn, a plant of the Magnoliophyta branch and the great family of Euphorbiaceae,[3] This plant has potential pharmacodynamic properties such as antidiabetic drugs[4,5], anticonvulsant[6], antileptospiral activity[7], antimicrobial activity[8,9,10], antinociceptive activity[11]; anti-inflammatory activity.[12,13] *P. amarus* is a recurrent plant used in the treatment of hepatitis in Benin.[14] Recent studies have focused on the evaluation of the antioxidant activity of *P. amarus* after carrying out it's polyphenolic compounds composition and showed that the ethanolic extract of *P. amarus* provide the best antioxidant activity.[15] The objective of our study is to evaluate the hepatoprotective and nephroprotective properties of *P. amarus* against hepatotoxicity and nephrotoxicity induced by carbon tetrachloride on in vivo model of Wistar strain rats.

MATERIAL AND METHODS
Plant material
The stem leaves powder of *P. amarus* (EEPh) were harvested at Covè (Latitude 7° 13' 8" N, Longitude 2° 20' 22"E, Altitude 102 m), (Department of Zou, Benin) in July 2015 and identified under the number AA 6552 / HNB in the national herbarium of Benin.

Preparation of ethanolic extract of *P. amarus* (EEPh)
The collected stem leaves powder of *P. amarus* were shade-dried and powdered in a mixer-grinder to get a coarse powder. A quantity of 600g of the powder of the leaves is soaked and macerated in 3l of ethanol, under gentle agitation for one night at room temperature)

forming a maceration. Ethanol extract is recovered after filtration using a paper filter; ethanol is eliminated from the filtrate by evaporation under reduced in a rota-evapour pressure.

Treatment of animals

Wistar albinos rats (142g-200g), aged 10 to 15 weeks were obtained and acclimatized in the Laboratory of Animal Physiology and Experimental Pharmacology of the Faculty of Science and Technology of the University of Abomey-Calavi two weeks before the beginning of the experiment at a constant temperature of 22 ± 1°C with a 12-hour cycle of light and 12 h of darkness. They are fed with granular feed and ad libitum water without discontinuity in feeding bottles. Carbon tetrachloride, supplied by UBC.HR. Leuven 6172 Belgium, is used for the induction of hepatic and renal poisoning. The Belle France extra virgin olive oil (Francap, BP 30403-75564 Paris Cedex 12) is used to prepare the poisoning solution. The Legalon ® (lot B 0902953, MADAUS GmbH 51101 Cologne-Germany) used as liver reference product contains 70 mg of silymarin (SIL). The animals were taken care as per OCDE guidelines and the experimental protocol was approved by animal ethics committee of Animal Physiology department of Abomey-Calavi University (Benin).

Hepatoprotective activity

Ten batches of six Wistar rats were randomized. They are individually marked and then kept in their cages for acclimation to laboratory conditions for two weeks before the experiment. All rats are weighed before the experiment. They received carbon tetrachloride (CCL_4, 1ml/kg diluted 1/5 in olive oil) to induce toxicity in which the liver and kidney are the primary target organs (hepatotoxicity and nephrotoxicity). Belle France olive oil (HO) is used to prepare CCL_4 solution. The CCL_4 were administred intraperitoneally and the animals were treated curatively with the ethanolic extract of *P. amarus* (EEPh) according to the model of.[16]

- batch A (negative control): rats received distilled water
- batch B (negative control): rats received olive oil
- batch C (positive control): rats received 1 ml/kg CCL_4 (1:5) without treatment
- batch D (reference): rats received 1 ml/kg CCL_4 (1/5) and treated with (SIL) at 300 mg/ kg PV

Test batch 1: rats given 1 ml/kg CCL_4 (1/5) and treated with EEPh at 250 mg / kg PV
Test batch 2: rats given 1 ml/kg CCL_4 (1/5) and treated with EEPh at 500 mg / kg PV
Test batch 3: rats given 1 ml/kg CCL_4 (1/5) and treated with EEPh at 750 mg / kg PV

The batch C don't received corrective therapy (Positive Control) while batch D received silymarin (300 mg/kg). The batches 1, 2 and 3 received respectively 250 mg/kg, 500 and 750 mg/kg of *P. amarus* extracts orally once daily for 7 days. On day 8, the blood was taken by retroorbital puncture in dry tubes using a micropipette with unheparinized hematocrit. The blood samples were centrifuged at 3000 tr/min for 15 minutes. The serum collected was used for the determination of the various biochemical parameters.

Body weight

The individual weight of each rat is determined one hour before administration of the test substance and then at least once a week. The weight changes are calculated and recorded.

Biochemical examinations

Portions of the blood are taken from all rats by retro-orbital puncture 24 h after the last extract administration, biochemical examinations at the Laboratory of Applied Biology Research of the Abomey-Calavi Polytechnic School. The biochemical tests are carried out by the kinetic method according to the methodology of[17] using the Semi-automate brand RAYTO. These include the determination of transaminases (ASAT, ALAT), alkaline phosphatase (PAL), bilirubin (free and conjugated), urea, total protein, creatinine.

Statistical analysis

All data is processed using Microsoft Excel 2010 and were analyzed by One-Way Analysis of the variance (ANOVA) followed by Tukey's post-test for the comparison of the averages. All analyses were performed using the statistical program Minitab version 16.FR. The threshold of significance is 5%.

RESULTS AND DISCUSSION

Morphometric parameters

The animals of the different lots are weighed before and after the treatments. The weights at the beginning and at the end and their variations are summarized in Table 1.

Table 1: Average weights of Wistar rats at the beginning and at the end of treatments.

Traitment	Initial average weight	Final average weight	Change average weight
(Control HO)	147.00 ± 11.08^{bc}	143.67 ± 14.49^{e}	4.00 ± 3.03^{b}
(Control H_2O)	164.50 ± 12.11^{cd}	158.67 ± 11.69^{bcd}	7.17 ± 4.83^{b}
CCL_4	170.40 ± 36.09^{ab}	128.20 ± 15.01^{f} *	34.83 ± 27.72^{a}
CCL_4-SIL	185.00 ± 7.21^{a}	177.60 ± 14.22^{a}	8.50 ± 11.10^{b}
CCL_4-EEPh (250mg)	163.00 ± 14.97^{cd}	165.40 ± 8.38^{ab}	10.00 ± 7.92^{b}
CCL_4-EEPh (500mg)	145.60 ± 9.53^{d}	147.00 ± 6.40^{cde}	2.83 ± 2.86^{b}
CCL_4-EEPh (750mg)	146.17 ± 8.73^{cd}	146.67 ± 8.19^{cde}	4.50 ± 4.14^{b}

In the same column the averages that do not share a letter are different.
*: Significant statistical difference. (P <0.05).

Except the batches witch received CCL_4 and CCL_4-EEPh (750mg), all the other batches showed an insignificant weight change. The batch that received CCL_4 showed a significant decrease in weight whereas the batch treated with EEPh (750mg) showed a slight insignificant gain weight. The fall in weight at the level of the CCL_4 batch would probably be related to the toxic effects of CCL_4. This confirms the safety of *P. Amarus* in accordance with previous i*n vivo* toxicity studies carried out on this plant. These results are similar to those of[18] and[19], who found a loss in weight during the evaluation of the hepatoprotective activity of *Gomphrena celosioides* and an insignificant gain along the evaluation of the hepatoprotective activity of the ethanolic extract of *Cinnamomum zeylanicum* L.

Biochemical Examinations

The various biochemical parameters explored have informed about the probable effects of EEPh in the liver and kidney. The transaminases (ALAT and ASAT), alkaline phosphatase (PAL), bilirubin (free and conjugated), blood glucose are parameters of the liver while uric acid, creatinine and total proteins are kidney parameters. The results of the various assays are shown in the following tables.

Table 2: Effects of *P. amarus* on the hepatic parameters of the control batches (negative and positive) and of the test batches (intoxicated and treated batches). These results represent mean ± SEM (standard error of mean).

Hepatic parameters / batches	ASAT/ GOT	ALAT/GPT	BT	BC	PAL
(Control HO)	48.12 ± 15.97 ^{bc}	38.03 ± 13.39^{cd}	4.08 ± 0.62^{e}	3.27 ± 0.83^{cd}	32.07 ± 4.52^{bc}
(Control H_2O)	37.00 ± 8.81^{cd}	30.00 ± 5.97 ^{d}	3.89 ± 0.67^{e}	2.55 ± 0.75^{d}	25.67 ± 7.94^{c}
CCL_4	140.50 ± 14.89^{a} *	150.63 ± 17.20^{a}*	14.07 ± 1.407^{c} *	8.10 ± 1.96^{ab}*	69.82 ± 23.59^{a} *
CCL_4-SIL	33.17 ± 8.01^{cd}	32.82 ± 9.91^{cd}	3.38 ± 1.02^{e}	3.28 ± 1.52^{cd}	63.27 ± 23.33^{a}*
CCL_4EEPh(250mg)	55.91 ± 24.76^{bc}	71.19 ± 24.22 ^{b}*	9.57 ± 3.38^{d}*	5.75 ± 1.28 ^{bc}*	59.27 ± 6.492^{a} *
CCL_4-EEPh (500mg)	48.17 ± 5.25^{bc}	34.72 ± 9.72 ^{cd}	4.76 ± 0.76^{e}	3.17 ± 1.13 ^{cd}	62.88 ± 9.75^{a}
CCL_4-EEPh(750mg)	16.69 ± 2.66^{d}*	33.92 ± 1.72 ^{cd}	2.35 ± 0.92^{e}*	2.85 ± 0.76^{cd}	76.42 ± 4.5

In the same column the averages that do not share a letter are different.
*: Significant statistical difference. (P <0.05).

Table 3: Effects of *P. amarus* on renal parameters. Control batches (negative and positive) and test batches (intoxicated and treated batches). These results represent the mean ± standard deviation

	Urea	Creatinine	Totals proteins
(Control HO)	0.37 ± 0.06 ^{cd}	18.82 ± 2.99 ^{bc}	57.10 ± 4.33 ^{bcd}
(Control H_2O)	0.35 ± 0.03 ^{d}	17.39 ± 1.47 ^{bc}	58.62 ± 17.36^{d}
CCL_4	0.50 ± 0.06 ^{abc}*	27.08 ± 4.97 ^{a}*	47.74 ± 4.48^{d}*
CCL_4-SIL	0.38 ± 0.03 ^{bcd} -----	16.53 ± 3.00 ^{bc} -----	53.49 ± 3.08^{cd} -----
CCL_4-EEPh(250mg)	0.56 ± 0.08 ^{a} *	18.10 ± 1.39 ^{bc}	57.49 ± 6.67^{abcd}
CCL_4-EEPh(500mg)	0.55 ± 0.02 ^{a}*	17.35 ± 3.08 ^{bc}	61.26 ± 4.48^{ab}
CCL_4-EEPh(750mg)	0.25 ± 0.06 ^{d}*	14.56 ± 1.25 ^{c}*	73.14 ± 12.01^{abcd}*

In the same column the averages that do not share a letter are different.
*: Significant statistical difference. (P <0.05).

The Figure 1" shows the effects (expressed as a percentage) of EEPh on transaminases and bilirubins.

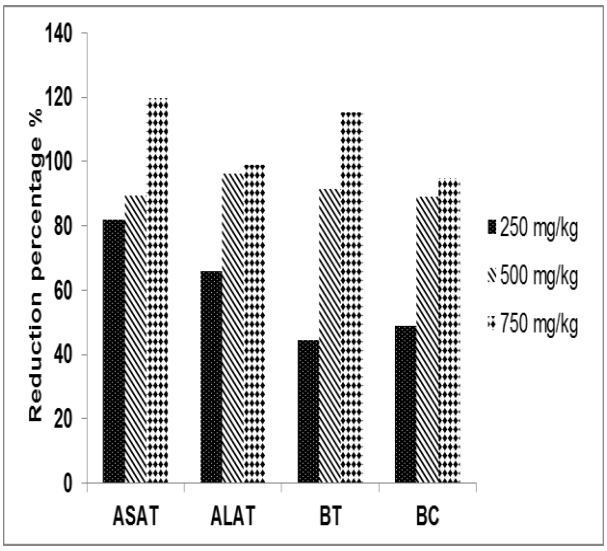

Figure 1''. Effects of different doses of EEPh on ASAT, ALAT, total and conjugated bilirubin The Figure 2" shows the effects (expressed as a percentage) of EEPh on alkaline phosphatases, urea and creatinine.

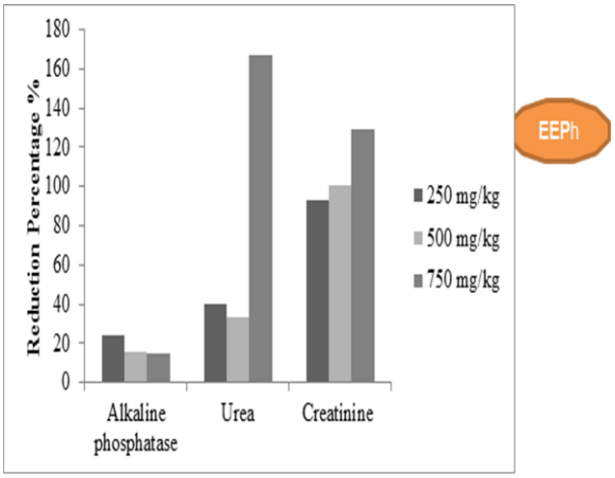

Figure 2''. Effects of different doses of EEPh on alkaline phosphatase, urea and creatinine The Figure 3" shows the effects (expressed as a percentage) of EEPh on total proteins.

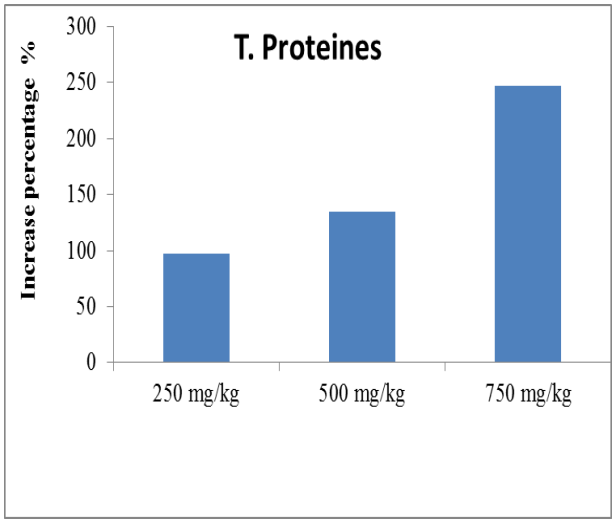

Figure 3". Effects of different doses of EEPh on total proteins

Table 4: Mean Percentages of Reduction in CCL_4 Toxicity (1 ml/kg i.p diluted 1/5)

	Mean Percentages of Reduction (hepatic parameters)	Mean Percentages of Reduction of renal parameters(Urea and creatinine)
EEPh (250) mg	51.6 %	66.33 %
EEPh (500) mg	76.25 %	66.87%
EEPh (750) mg	88.20 %	147.93 %

The results show that there is no significant difference between the liver and renal parameters of the two negative controls (water, olive oil). The olive oil (HO) used as vehicle for the dilution of CCL_4 has not effect on the physiology of the rats and may have a protective effect by causing an increase in the activity of the antioxidant enzymes and decreased signs of damage to the liver.[20,21]

At the beginning of its biotransformation the CCL_4 undergoes a reductive deshalogenation reaction catalyzed by P_{450} to give the trichloromethyl free radical (CCL_3).

The highly reacted CCL_3 formed readily interacts with the molecular oxygen to form the peroxyl trichloromethyl radical (CCL_3OO).[22] These radicals bind to proteins, lipids or abstract a hydrogen atom of an unsaturated lipid to cause lipid peroxidation and lesions, thus contributing significantly to the pathogenesis of diseases.[23] The toxicity of CCL_4 is mainly due to the appearance of free radicals or toxic forms of oxygen which induce lipid peroxidation leading to the destruction of cell membranes.[24] This is a mandatory and predictable indirect toxicant.[25], [26] The increase in serum levels of transaminases and alkaline phosphatases after CCL_4 injection is evidence of significant hepatic involvement. CCL4-induced liver lesions are commonly used as a model for liver drug screening and the extent of damage is assessed by the level of cytoplasmic transaminases (ALT and ASAT) and circulating APL.[27,28]

The test lot which received only CCL_4 exhibited a significant increase in transaminases (ASAT, ALAT), alkaline phosphatase (PAL) as well as bilirubin (total and conjugated) of urea, creatinine and a significant decrease in proteins Total. Increased serum levels of ALT and AST in CCL_4-mediated rats is an indication of the damaged structural and functional integrity of liver cell membranes since these cytosolic enzymes are released into the circulation after cellular lesions hepatic function.[29] The Carbon tetrachloride, besides exerting its toxic effect on liver, also reportedly gets distributed at higher concentrations in the kidney than in the liver.[30] The mechanism of CCL_4 renal toxicity is almost the same as that of liver, but the cytochrome P-450 predominantly[31,32] shows a high affinity to the kidney cortex. The CCL_4 caused hepatorenal injury and the transport function of hepatocytes and nephrotic cells gets disturbed in the leakage of plasma membrane, thereby causing an increased enzyme level in the serum.[33] The test batch, which has received only CCL4 exhibited a significant increase in transaminases (ASAT, ALAT), alkaline phosphatase (PAL), as well as bilirubin (total and conjugated) of urea, creatinine and a significant decrease in proteins. The variation of hepatic and renal parameters recorded the extensive disruption of the structure and function of the liver and kidney. Silymarin has hepatoprotective properties and is used in various liver diseases.[34] Various studies indicate that Silymarin exhibits strong antioxidant activity[35] and shows protective effects against hepatic toxicity induced by a wide variety of agents by inhibiting lipid peroxidation[36,37,38,39], while antioxidant activity has also been linked to the hepatoprotective effect of some extracts. Our results are similar because the batch received silymarin after CCL_4 intoxication shows that the liver and renal parameters show no significant difference compared to the negative control except for alkaline phosphatases. The analysis of the results of the test batches having received different doses of EEPh extracts show that the dose of 500 mg/kg prevents the appearance of the lesions in the liver because the levels of AST, ALT, BT and BC show an insignificant statistical difference versus negative control with percentages of respective reductions of 89.20%, 96.08%, 91.45%, 88.82% versus 103.7%; 97.66%; 106.15%; 86.84% for silymarin. The hepatic lesions induced by free radicals can be prevented or corrected by antioxidants.[40] EEPh has a dose-dependent hepatoprotective effect on these liver parameters. Concerning renal parameters, the 750 mg/kg dose seems to impede the appearance of lesions in the kidneys because this dose significantly lowers the urea and creatinine levels and increase that of the total proteins with respective following percentages (reduction of 166.66%, 129.20%; and an increase of 247.56%). Our data are consistent with those of[41] and[42] who showed that a single intraperitoneal administration of CCL_4 (1.5 ml /kg bw of 20% CCL_4 in olive oil) raised serum levels of creatinine and urea. The observed increase is indicative of altered glomerular function and renal disorder.[43] Specifically, the increase in creatinine suggests that muscle wastage occurred during CCL_4 intoxication since creatinine production has a direct relationship to muscle mass.[44] As a result, muscle proteins are depleted and increasingly desaminated, but associated kidney disorders prevent the normal excretion process and thus cause accumulation and elevation of serum urea and creatinine levels. Indeed, the dose, duration and route of administration determine the extent of kidney damage during CCL_4 poisoning. These results are comparable to those of[45] who have shown that the ethanolic extract of *Homalium letestui Pellegr* (Flacourtiaceae) has protective properties.

CONCLUSION

The study showed a significant protective effect of *P. amarus* ethanolic extract against CCL_4-induced hepatotoxicity and nephrotoxicity. The ethanolic extract of *P. amarus* protected the liver and kidney against the oxidative stress of CCL_4 at 500 mg/kg and 750 mg/kg, respectively. *P. amarus* ethanolic extract maintained this hepatoprotective and nephroprotective through lipid peroxidation improvement by its scavenging activity of free radicals and enhancement of the antioxidant defense systems.

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