RESEARCH ARTICLE

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CONFLICT OF INTEREST NONE DECLARED

Phyllanthus amarus Schumach. & Thonn : Effects of Ethanolic Extract of Stem Leaves on Wistar Rats Liver and Kidney Functions

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ABSTRACT

Phyllanthus amarus Schumach. & Thonn is used traditionally in the treatment of liver diseases. The main objective of our study is to evaluate the toxicity or the effects of the acute oral administration of the ethanol extract of this plant. Exploratory tests for acute oral toxicity are performed *in vivo* on Wistar albino rats in a limit test of 2000 mg/kg in accordance with the OECD Guidelines. The various clinical signs observed after administration and for 14 days were recorded and no mortality was observed. With the exception of red blood cells and hemoglobin, whose statistical difference from the control was significant ($p < 0.05$), all other haematological parameters showed a insignificant statistical difference to the control ratio ($p > 0,05$). Concerning the biochemical parameters, except glycemia and the total proteins of the control batches which show a significant statistical difference after 14 days ($p > 0.05$), all the other biochemical parameters show insignificant statistical difference in the test batches and Controls ($p > 0.05$) as well as the weight variation of the animals. The ethanolic extract of *Phyllanthus amarus* therefore has no toxic effect on the biochemical and haematological parameters studied at a dose of 2000 mg/kg. The lethal dose of the ethanolic extract of *Phyllanthus amarus is greater than 2000 mg/kg.*

Keywords: *Phyllanthus amarus* Schumach. & Thonn, extract, toxicity, oral, acute, lethal.

Introduction

Phyllanthus amarus Schumach. & Thonn is from the branch of the Magnoliophyta and the large family of Euphorbiaceae, APG (2009). Euphorbiaceae is one of the largest plant families in the world (326 genus, 7750 species). *P. amarus* is used traditionally to treat hepatitis (Tássia Campos de Lima Silva et *al.*, 2012, Guinnin et *al.*, 2015). Plants of the genus Phyllanthus are widely distributed in most tropical and subtropical countries and are generally used in traditional medicine to treat chronic liver disease (Liu et *al.*, 2003).Still called bitter Phyllanthus (French); Henlenwe (fon); Ashasha (yoruba, nagot); Sobaru (bariba); Banna banna biriku (dendi) (Akoègninou et *al.*, 2006), *P. amarus* is an erect grass up to 50 cm tall, glabrous. The stem and leaves are light green; the flowers and fruits are small and hanging at the tips of the twigs. This species flowers and then fructifies between March and October. *P. amarus* is a pantropical species found in forest galleries, fallows, and roadside. In addition to its hepatoprotective activity, this plant is used as a diuretic, astringent (Patel et *al*, 2011) also in the treatment of diabetes (Povi Lawson-Evi et *al,* 2008). The phytochemical analysis of the *P. amarus* extract confirmed the presence of tannins, saponins, flavonoids and alkaloids. The plant extract contains high levels of saponins, tannins, flavonoids and alkaloids (Naaz, 2007, Krithika and Verma, 2009). Despite the work done on the pharmacological properties of this plant, there is a lack of information on the toxicity of the ethanol extract of this plant. The main objective of this work is to evaluate the acute oral toxicity of the ethanol extract of this plant.

Material and Methods

Plant material

The plant material was the ethanolic extract of the leafy stem powder of *P. amarus* (EEPh). The stem leaves 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.

Animal testing equipment

Acute oral toxicity tests were performed on Porton-Wistar (140g-174g) rats, aged 9-12 weeks randomly selected. The rats come from the Laboratory of Animal Physiology of the Faculty of Science and Technology of the University of Abomey-Calavi and are acclimatized in the laboratory of the Laboratory of Research in Applied Biology at least two weeks before the beginning of the experiment at a constant temperature of 22 ± 1 ° C with a cycle of 12 hours of light and 12 hours of darkness. They are fed with granulated feed and ad libitum water without discontinuity in feeding bottles.

Exploratory tests of in vivo toxicity of extracts

The tests will be carried out in accordance with the Guideline of the Organization for Economic Co-operation and Development (OECD) for the testing of chemicals through Method 423 (OECD, 2001). The principle of this test will be that with a sequential process, using a minimum number of animals in stages, information on the acute toxicity of these plants will be obtained and sufficient for the classification needs. The extracts of each plant will be dissolved in physiological water and administered to the rats at a rate of 1 ml / 100 g of body weight. Control rats were given extracts of physiological water instead. They are marked for individual identification. A limit test at a dose level of 2000 mg/kg was chosen because information indicating that the leafy stem of *P. amarus* is not likely to be toxic is available, i e toxicity is likely above the prescribed dose limit. The rats were divided into two batches of three rats after blood tests to ensure homogeneity of batches. Control batch (A) received no extract but distilled water while batch (B) received 2000 mg/kg of ethanolic extract of *P. amarus*. The animals were observed individually at least once during the first 30 minutes and at least twice during the first 24 hours after treatment. Particular attention was paid to them daily for 14 days after administration of the substance. All observations were systematically recorded. Particular attention has been paid to observing the various manifestations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

The following parameters will be searched: BodyWeight

The individual weight of each rat will be determined one hour prior to the administration of the test substance and then at least once a week. Weight changes will be calculated and recorded. At the end of the test, the animals will be weighed and sacrificed by an overdose of anesthesia.

Blood and Biochemical Examinations

Blood sample were taken from all rats by retro-orbital puncture for hematological and biochemical examinations at the Applied Biology Research Laboratory of the Abomey-Calavi Polytechnic School. The hematological examinations were carried out using a SYSMEX KX-N21 automaton according to the method used by Sodipo et *al.* (2012). These include the enumeration of red blood cells, white blood cells, platelets and determination of hemoglobin, hematocrit, mean globular volume, average corpuscular content in hemoglobin, and average corpuscular concentration in Hemoglobin.

Biochemical examinations will be carried out by the kinetic method in accordance with the methodology of Sodipo et *al.* (2012) using the Semi-Automate brand RAYTO. These include the determination of transaminases (ASAT, ALAT), alkaline phosphatase (PAL), bilirubin (free and conjugated), blood glucose, urea, total protein, creatinine.

Statistical Analyzes

All data is processed using Microsoft Excel 2010 and Minitab version 16.FR. The latter was used for the analysis of the variance (ANOVA with a comparative factor) for the comparison of the averages. The threshold of significance is 5%.

Results and Discussion

Clinical signs observed

After administration of the extracts the animals were observed individually at least once during the first 30 minutes and at least twice during the first 24 hours after treatment. Particular attention was paid to them daily for 14 days after administration of the substance.A few minutes after the injection of the ethanolic extract of the *P. amarus* leaf stem powder (EEPh), we recorded a short agitation period of about 2 to 3 minutes in almost all the batches and then the animals have resumed their normal habit. Then all observations were recorded systematically and summarized in the following table:

The *in vivo* oral toxicity tests of the ethanolic extract of *P. amarus* performed in a limit test of 2000 mg/kg did not cause any mortality and the various clinical signs appeared reversible and disappeared after two weeks. Similar results have been reported by (Povi Lawson-Evi et *al*, 2008) which, by studying the acute and subacute

oral toxicity of aqueous and hydro alcoholic extracts of P. *amarus,* observed no mortality.

Lots Clinical Signs	Lot(I)	Lot(II)
Salivation	-	-
Accelerated breathing	-	+
Tremors	-	-
Sleep	+	+
Diarrhea	-	-
Lethargy	-	+
Paralysis	-	-
Abdominal constrictions	-	+
Comma	-	-

- : absence of signs +: Presence of signs

Table 1 - Clinical signs observed 24 hours after and during the 14 days after injection of the ethanolic extract of *P. amarus* Schumach. & Thonn.

These results confirm those of Shirish S. Pingale et *al.*, 2011 and Tássia Campos de Lima Silva et *al.*, 2012 who worked on the aqueous extract of the same plant and notes that it did not cause any mortality even at doses greater than 2000 mg/kg.

Effect of the ethanolic extract of P. *amarus* (EEPh) on the weight of Wistar rats and their organs

Analysis of the variation in body weight at the level of the batch obtained with the ethanolic extract of *P. amarus* (EEPh) and the control batch shows a slight weight drop 7 days after treatment (FIG. 1). This variation is not significant (p> 0.05) so it is certainly to other factors such as stress and nervousness of animals during and after collection. The ethanolic extract of *P. amarus* therefore has no effect on the variation of the weight of the animals. On the other hand previous work has shown that the presence of polyphenols such as tannins can be responsible for poor assimilation of food and can promote a reduction in weight. These results corroborate the work of (Povi Lawson-Evi et *al.*, 2008) who, by studying the acute and subacute oral toxicity of aqueous and hydroalcoholic extracts of *P. amarus*, find that the weight difference between the control and the treated batches is not significant.In addition, variation in body weight is used as an indicator of the adverse effects of chemical compounds (Hilaly et *al.*, 2004).This reduction in weight can be explained by a reduction in the consumption of food, but also by the possibility of dose / absorption interactions and by the reduction in the amount of food absorbed. Other studies have also shown a reduction in the weight of rats after oral administration of the *Chicococca alba* extract (Gazda et *al.,* 2006) and that of *Stryphnodendron adstringens* (Rebecca et *al.*, 2002).

The averages obtained are shown in Fig1. and in Fig 2. There is a variation in average weights over time.

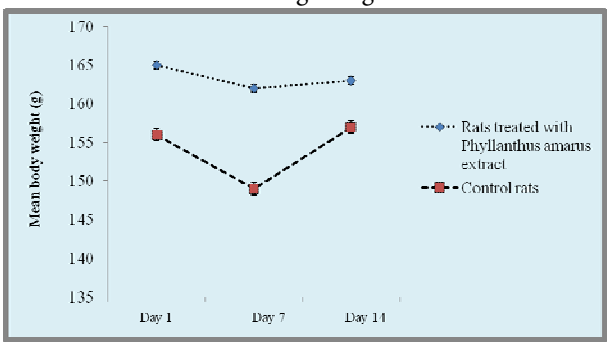

Fig.1-Effect of the ethanolic extract of P. *amarus* (2000mg/kg) on the mean weight of Wistar rats

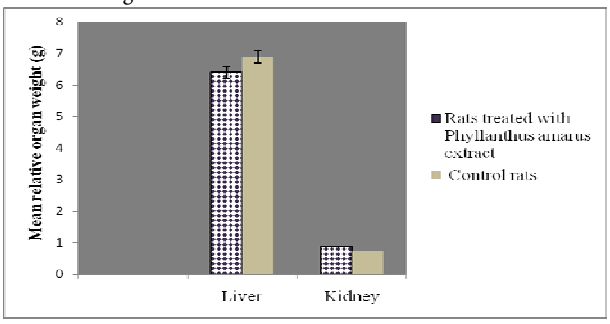

Fig 2- Effect of the ethanolic extract of *P. amarus* (2000mg/kg) on the average weight of the organs.

Blood and Biochemical Examinations

The results of the haematological examinations are summarized in the following table

Batch Parameters	Batch I	Batch II
GB (10^9 / L) *	5.35 ± 0.07	8.4 ± 1.27
HGB (g/dl) **	14.45 ± 0.35	11.00± 0.28
GR (10^{12}/ L) **	8.380 ± 0.255	6.42 ± 0.12
HCT (%) *	42.500 ± 3.536	35 ± 1.41
VGM (fL) *	51.850 ± 1.061	54.65 ± 0.91
TMH (pg) *	16.100 ± 1.414	17.05 ± 0.21
CCMH (g/dL)*	31.850 ± 1.061	30.6 ± 0.07
IDR-CV (%) *	11.200 ± 2.970	11.65 ± 0.35
IDR-DS (fL) *	24.500 ± 1.414	25.95 ± 0.63
PLT (10^9/L) *	534.00 ± 19.80	544 ± 2.82
VMP (fL) *	7.700 ± 1.697	8.05 ± 0.07
IDP *	13.550 ± 1.768	13.9 ± 0.56
PCT (%) *	0.6090 ± 0.1117	0.44 ± 0.01

Table 2- Effect of the ethanolic extract of *P. amarus* (EEPh) on blood haematological parameters.

**: Significant statistical difference between Lot II and Lot I Control for the parameter considered (p <0.05)

*: Statistical difference not significant (p> 0.05); M ± esm = mean ± standard error

On average, n = 3, GB = white blood cells, HGB = hemoglobin, GR = red blood cells, HCT: Hematocrit, VGM = mean globular volume;

TMH = mean hemoglobin content; CCMH = mean corpuscular hemoglobin concentration; PLT = platelets; IDR = red blood cell distribution index; VMP = mean volume of platelets; IDP = Platelet distribution index.

It is noted that, with the exception of red blood cells and hemoglobin, whose statistical difference from the control is significant (p <0.05), all the other haematological parameters present a statistical difference that is not significant compared to the control (P> 0.05). The absence of the variation in hematocrit leads us to rule out the hypothesis of hemoconcentration. On the other hand, the decrease in red blood cell count and hemoglobin may suggest that the ethanolic extract of *P. amarus* has a hemolytic action, confirming the work of Taiwo IA, 2009 and Joseph B, 2011 who treated albino rats with aqueous extract of P. *amarus* (leafy stem) an experimental studies found that this produced a dose-dependent decrease in the level of erythrocytes and globular volume. This may also be due to the blood sampling conditions and also to physiological hemolysis of the aged red blood cells at the end of their lifetime. Our results are contrary to those of Maduka et *al.* (2003) and Mama KONE et *al.* (2009), who showed that the total aqueous extract of *Sacoglottis gabonensis* does not cause changes in the erythrocytic lines. Indeed, Maduka et al. (2003), studying the influence of *Sacoglottis gabonensis* on the side effects of 2,4-dinitrophenylhydrazine on blood and cellular metabolism showed that administration of *Sacoglottis gabonensis* to rats did not alter the levels of globules Red, hemoglobin, hematocrit, white blood cells, lymphocytes, neutrophils and monocytes. Several factors may explain this difference. Indeed, the chemical composition of *Phyllanthus amarus* certainly differs from that of *Sacoglottis gabonensis* and even though it is the same plant, Mama Kone et *al.* (2009) confirm that the composition of a plant in secondary metabolites varies according to the geographical location, the sample taken, the period, the time of sampling and the storage conditions.According to the author the secondary metabolites are responsible for the bioactive properties of plants.

The biochemical parameters explored have informed us about the likely effects of the ethanolic extract of *P. amarus* on the liver and kidney. Transaminases (ALAT and ASAT), alkaline phosphatase (PAL), bilirubin (total and direct), blood glucose are parameters of the liver while urea, creatinine and total proteins are kidney parameters. The results of the various assays are shown in the figures ranging from 3 to 8.

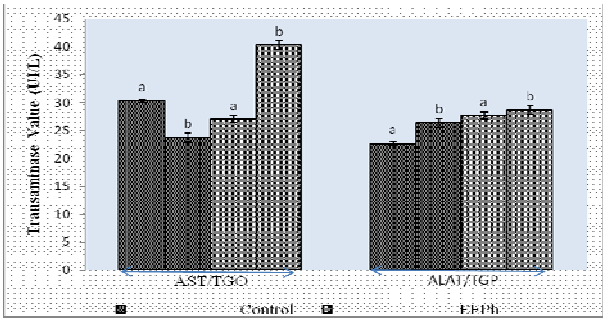

Fig.3- Effect of the ethanolic extract of *P. amarus* (2000mg/kg) on transaminase. (a : 24h ; b : day 14)

The ASAT and ALAT transaminase of (lot II) treated with EEPh and of the control lot (lot I) showed no significant difference (p> 0.05) after 14 days

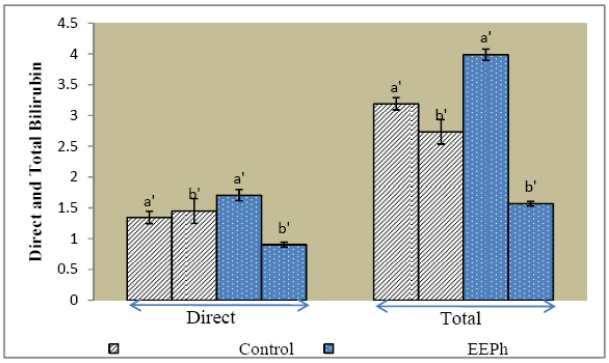

Fig 4- Effect of the ethanolic extract of *P. amarus* (2000mg/kg) on direct and total bilirubin

a' :24h ; b' : day 14

The direct and total bilirubin of (lot II) treated with EEPh and the control lot (lot I) showed no significant difference (p> 0.05) after 14 days.

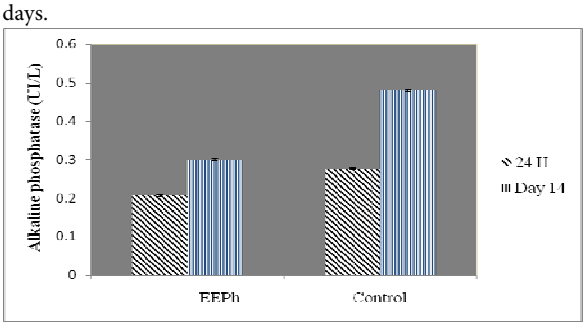

Fig 5- Effect of the ethanolic extract of *P. amarus* (2000mg/kg) on alkaline phosphatase (PAL)

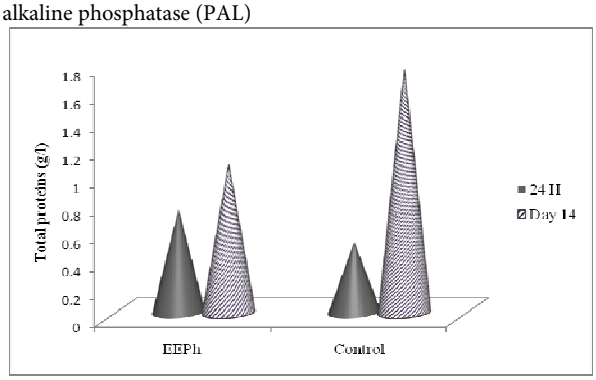

Fig 6- Effect of the ethanolic extract of. *P. amarus* (2000mg/kg) on total proteins

The total protein (lot II) treated with EEPh showed no significant difference (p> 0.05) after 14 days, whereas those of the control (lot I) group showed a significant difference (p <0.05).

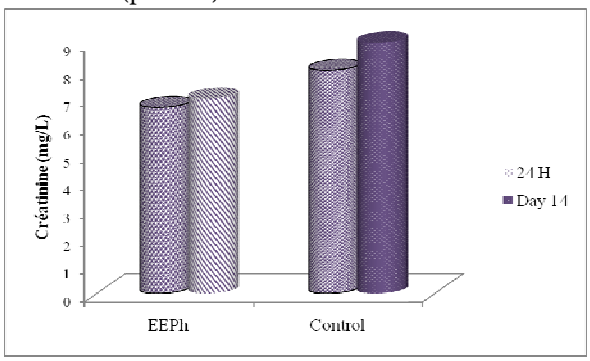

Fig 7- Effect of the ethanolic extract of P. *amarus* (2000mg/kg) on Creatinine

Creatinine (lot II) treated with EEPh and control (lot I) showed no significant difference (p> 0.05) after 14 days.

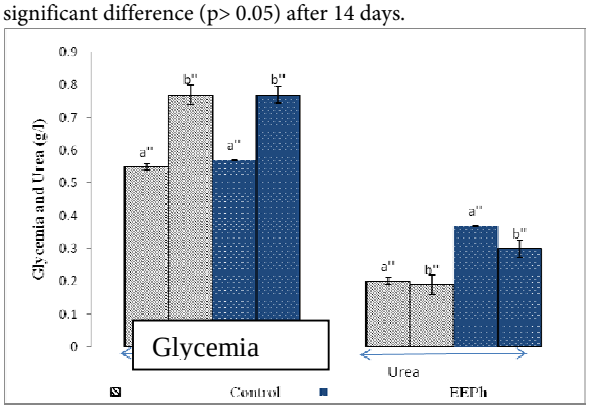

Fig 8- Effect of the ethanolic extract of *P. amarus* (2000mg/kg) on blood glucose and urea

a" : 24h ; b" : day 14

There was no significant difference (p> 0.05) in glucose (lot II) treated with EEPh after 14 days, whereas that in the control batch (lot I) showed a significant difference (p <0.05).The urea of (lot II) treated with EEPh and the control lot (lot I) showed no significant difference (p> 0.05) after 14 days.

The results show that, with the exception of blood glucose (Fig. 8) and total proteins (Fig. 6) of the control batch, whose contents 24 h after administration and 14 days have a significant difference (p < 0.05), other biochemical parameters such as ALAT / TGP and ASAT / TGO transaminases (Fig.3), total and conjugated bilirubins (Fig.4), alkaline phosphatases (Fig. Creatinine (Fig.7), urea (Fig.8), showed an insignificant statistically difference (p> 0.05) in test batch (II) and control batch (I). The variations in glycemia and total proteins in the control batch may be related to the result between inputs (food, synthesis, mobilization of reserves) and outputs (storage, catabolism, elimination). The ethanolic extract of *P. amarus*. Has no effect on plasma biochemical parameters.

From these results we can object that the ethanolic extract *P. amarus* proved to be non-toxic for the parameters tested, thus has no influence on the blood tissue and then on the vital organs such as the liver and the kidneys . This justifies practically the safety of this plant used in the treatment of hepatitis by some traditional healers and herbalists in Benin. These results corroborate those of S.K. Kushwaha et *al.*, Who carried out work on the acute oral toxicity of the standardized methanolic extract of *P. amarus*. Those authors noted no mortality and no significant change in the behavior of the biological animals. In addition, our results are similar to those of George Awuku ASARE et *al.*, Who have been studying the acute toxicity of aqueous leaf extract of *Phyllanthus niruri*, a plant of the same family and genus as *P. amarus*. The biochemical tests performed by these authors are bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin, globulin, alkaline phosphatase (ALP) - glutamyltranspeptidase (GGT), urea and creatinine and show no significant differences. Histological examinations will allow us to confirm these observations.

Conclusion

The ethanolic extract of the leafy stem of *P. amarus* administered orally at a dose of 2000 mg/kg has no toxic effect on the biochemical and hematological parameters studied. The lethal dose is therefore greater than 2000 mg/kg. These findings support its use in hepatitis treatment in some region of Benin. However, other studies such as histological examinations of the organs (liver and kidney) and chronic toxicity tests of *P. amarus* oral extracts deserve to be conducted in order to confirm its non-toxic character. The carrying out of the exploratory tests of cytotoxicity is imperative to certify the existence or not of possible secondary effects.

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