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Preliminary study of physiopathological changes associated with the ethanolic extract of the stem leaves of *Phyllanthus amarus* in rats

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The present work aimed to investigate physiopathological changes associated with the ethanolic extract of the stem leaves of *Phyllanthus amarus* (EEt(OH) *P.*a. Exploratory tests are performed *in vivo* on Wistar albino rats. Analysis of the variation in body weight at the level of the batch having received, and the control group showed a slight weight drop 7 d after treatment. This variation is not significant (p>0.05). The means relative organ weights show that there were no significant changes between control and treated groups (P > 0.05). No obvious clinical signs (tremor, breathing rate, paralysis). Slightly greater differences were noted for WBC of the Control group and rats treaded with EEt(OH) *P.a* (5000 mg/kg b.w.) on day 14 after the administration. These differences were statistically significant (p<0.05). Similarly, the platelet count was slightly lower for the control rats compared to the rats treaded. The various biochemical parameters explored, Glucose, Bilirubin (BIL), Alanine amino transferase (ALT), Aspartate amino transferase (AST), Total protein (TP), alkaline phosphatase (ALP), urea (URE), creatinine (CR), Total bilirubin (TBIL) and Indirect bilirubin (IBIL), have informed us about the probable effects of the stem leaves of *Phyllanthus amarus* in the liver and kidney. There was no significant differences (p>0.05) in glucose, total protein, urea, creatinine levels between control and experiemental groups at 14 d. Total bilirubin (TBIL) and Indirect bilirubin (IBIL), however, were statistically significant (p<0.05). Other biochemical parameters such as ALT, AST, ALP show statistically insignificant difference (p>0.05) in test and control batch.

Key words: *Phyllanthus amarus* Schum. & Thonn., physiopathological, breathing rate, paralysis, bilirubin, alanine amino, transferase, creatinine, alkaline, phosphatase, transaminases, aspartate amino.

INTRODUCTION

Plants have been the source of natural products used, since earliest times, in non-conventional medicine known as traditional medicine through communities worldwide.

Today, medicinal plants have continued to play an important role in the primary health care for more than 80% of people living in poor communities in the developing countries (Bennett and Brown, 2000; Nath et al., 2011). During the last decades, the use of medicinal plants in therapeutics has increased substantially (Castro et al., 2009; Lee et al., 2012) due to the increasing interests for natural substances. Among medicinal plants used in traditional medicine, some have antioxidant property and are used rightly or wrongly to prevent premature aging.

Although, the use of herbal medicine may be considered to be safe, some natural products are known to be toxic at high doses and others may have potential adverse effects after prolonged use. Many data concerning the safety of herbal medicine has been reported and frequently these reports are related to hepatotoxicity (Saad et al., 2006; Park et al., 2010) and nephrotoxicity (Cheng et al., 2006; Debelle et al., 2008). Hence, toxicological assessment of medicinal plants is required even if these plants have been used for centuries.

P. amarus belongs to the family *Euphorbiaceae* It is used in traditional medicine for gastrointestinal, kidney, liver, menorrhagia and other conditions (Patel et al., 2011).

Due to the worldwide usage of *P. amarus* in traditional medicine, this study was carried out to assess the physiopathological changes associated with the ethanolic extract of the stem leaves of *P. amarus* in rats.

The Beninese flora contains about three thousand species (Akoègninou et al., 2006), including *P. amarus Schumach.* and *Thonn*, a plant of the Magnoliophyta branch and the great family of Euphorbiaceae (APG, 2009). This plant has potential pharmacodynamic properties such as antidiabetic drugs (Evi et al., 2011; Kiran et al., 2011) anticonvulsant (Manikkoth et al., 2011), antileptospiral activity (Chandan et al., 2012), antimicrobial activity (Ushie et al., 2013; Saranraj et al., 2012; Oluwafemi et al., 2008), antinociceptive activity (Rajeshkumar et al., 2002) and anti-inflammatory activity (Adeolu et al., 2013; Kiemer et al., 2003).

In this study, acute oral toxicity activity of ethanolic extracts of *P. amarus Schumach. & Thonn*. stem leaves was investigated because of limited information available on its toxicity, despite the widespread use of this medicinal plant.

MATERIALS AND METHODS

Plant material

The plant material was the ethanolic extract of the leafy stem powder of *P. amarus.* The stem leaves were harvested at Abomey-Calavi Department of Littoral (Bénin), in July 2016 and identified under the number AA 6715/HNB in the National Herbarium of Benin

Preparation of ethanolic extract

Two hundred and fifty grams (250 g) of dry powder of the leafy stem of *P. amarus* were extracted by maceration with ethanol for 72 h stirring. Each extraction was repeated three times. The extracts were filtered and concentrated in rotary evaporator under reduced pressure to yield a thick green ethanolic extract. The obtained extracts were stored at 4°C until biological assay.

Ethical notice

The experimental protocol was approved by the Scientific Ethics Committee of the Doctoral School (Life Sciences) of the Faculty of Science and Technology (FAST) at the University of Abomey Calavi (UAC) under the number (UAC/FAST/EDSV/1112107).

Treatment of animals

Acute oral toxicity tests were carried out on randomly selected Wistar albino rats (140-160 g), aged 9 to 12 weeks. The rats are acclimatized to a constant temperature (22±1°C) and a 12 h light/dark cycle in the Laboratory of Physiopathologie Moléculaire et Toxicologie (Faculty of Science and Technology of the University of Abomey-Calavi) for two weeks. They were fed with granulated feed and *ad libitum* water without discontinuity in feeding bottles in light/dark cycle in the Laboratory of Physiopathologie Moléculaire et Toxicologie (Faculty of Science and Technology of the University of Abomey-Calavi) for two weeks. They were fed with granulated feed and *ad libitum* water without discontinuity in feeding bottles.

Acute oral toxicity

The tests were performed in accordance with the guidelines of the Organization for Economic Cooperation and Development (OECD) for the testing of chemicals substances through method 423 (OCDE, 2001). The ethanolic extract of this plant was dissolved in distilled water and administered to the rats at a ratio of 1 ml/100 g of body weight. Control rats were instead given distilled water. The rats were marked for individual identification. The rats were divided into two batches of six rats after blood tests to ensure homogeneity of batches. Control rats (six) did not receive extract but distilled water while the experimental animals (six) received 5000 mg/kg of an ethanolic extract of *P. amarus*. The animals were observed individually at least once during the first 30 min and at least twice during the first 24 h after treatment. Special attention was given to

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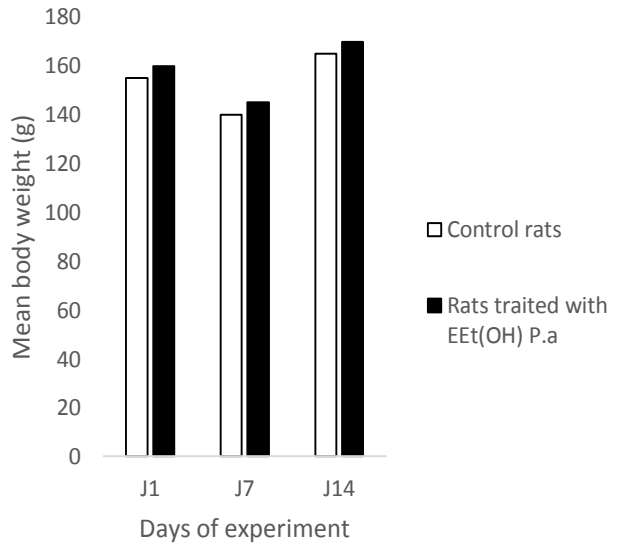

Figure 1. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on the average weight of wistar rats, n=6, results represent the means±SEM (standard error of mean).

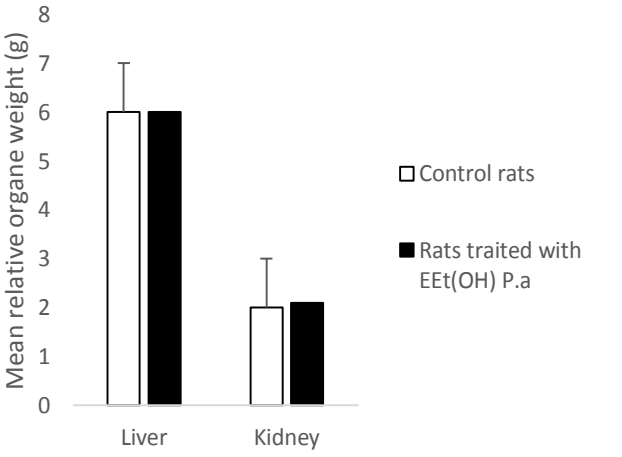

Figure 2. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on the average weight of the organs, n=6, results represent the means±SEM (standard error of mean).

Body weight

The individual weight of each rat is determined 1 h before administration of the test substance and then at least once a week. The weight changes were calculated and recorded.

Hematological indices

Portions of the blood are taken from all rats by retro-orbital puncture 14 days after the extract administration, for hematological examinations. Blood collection was done on live animals (without anesthesia), kept fasting for 16 h by puncturing the retro orbital sinus using a pasteur pipette previously rinsed with EDTA anticoagulant to 0.01%. The volume of collected blood was 0.5 to 2 ml. 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 were analyzed by using a diatron diagnostic abacus junior automatic hematology analyzer.

Biochemical analyses

The biochemical tests were carried out by the kinetic method according to the methodology of Sodipo et al. (2012) using the Semi-automate brand RAYTO. Portions of the blood were taken from all rats by retro-orbital puncture 24 h and 14 days after the extract administration. Blood is collected without anticoagulant in hemolysis tubes and centrifuged at 3000 rev / min for 15 min at a temperature of 4°C. The volume of collected blood was 0.5 to 2 ml. Following centrifugation of blood, the obtained serum was stored in Eppendorf tubes at a temperature of -4°C for the analysis of various biochemical parameters. These included the determination of total bilirubin (TBIL), direct bilirubin (DBIL), aspartate amino transferase (AST), alanine amino transferase (ALT), total protein (TP), alkaline phosphatase (ALP), urea (URE) and creatinine (CR).

Statistical analysis

Data collected from the biochemical and haematological analyses were expressed as mean ± SEM (n = 6). Statistical differences between treated groups and controls were determined by analysis of variance (ANOVA) followed by Fisher LSD test using Systat 10.0. Differences between groups were considered significant for $P < 0.05$.

RESULTS

Clinical signs observed

No obvious clinical signs (tremor, breathing rate, paralysis) were observed although quantitative assessments were no carried out.

Evolution of rat's weight and their organs during toxicity test

Animals were observed during 14 days and their weight was taken regularly. Figure 1 shows evolution of the rats weight during two weeks observation. Analysis of the variation in body weight at the level of the batch that received (EEt(OH) *P.*a, and the control group showed a slight weight drop 7 days after treatment (Figure 1). This variation was not significant ($p>0.05$). The means relative organ weights show that there were no significant changes between control and treated groups ($P > 0.05$) (Figure 2). At necropsy, no macroscopic change was observed in the internal organs in treated rats.

Hematological and biochemical parameters in rats

Hematological parameters did not show significant differences between control and rats treated with EEt(OH) *P.a* (5000 mg/kg b.w.) on day 14 after the

Table 1. Table of hematological indices of the Control group, and Rats treaded with EEt(OH) *P.a* (5000 mg/kg b.w.) on day 14 after the administration.

Blood hematological parameters	Control rats	Rats treated with EEt(OH) *P.a* (5000 mg/kg)
WBC × 10^3/ml	6.40±0.36	8.22±0.10 **
RBC × 10^6/ml	798.33±26.69	791.33±29.7*
HGB g/dl	6.9±2.6	6.8±2.0*
HCT %	11.4±0.2	11.07±0.8*
MCV fl	37.4±1.7	37.33±2.08*
MCH pg	30.7±0.4	30.1±8.3*
MCHC g/dl	21.8 ±0.40	22.50±0.35*
PLT × 10^3/ml	798.33±26.69	821.33±29.7**

n=3, results represent the means ± SEM (standard error of mean), *: Insignificant statistical difference (p>0.05); **: Significant statistical difference between Rats treaded with EEt(OH) *P.a* and control rats for the parameters considered (p<0.05), M ± esm = mean ± standard error on average, n = 3. EEt (OH) *P.a* **=** ethanolic extract of *Phyllanthus amarus Schumach. & Thonn*. NS = Not significant; WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelet.

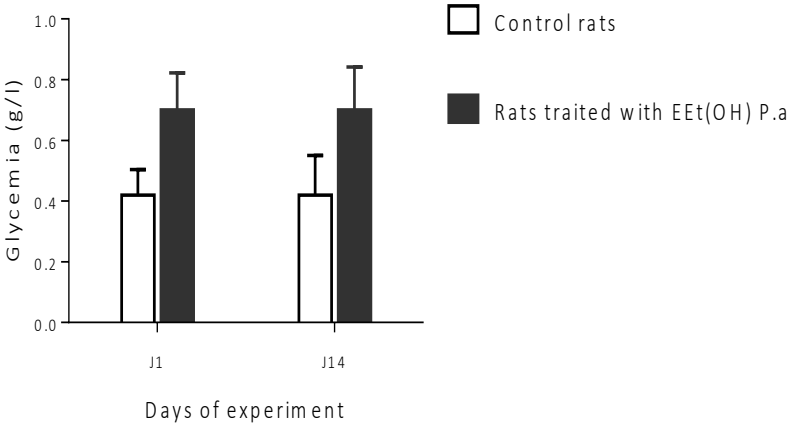

Figure 3. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on blood glucose, n=6, results represent the means±SEM (standard error of the mean).

administration (Table 1 and Figure 3 to 11). Slightly greater differences were noted for WBC of the control group (6.40× 10^3/ml) and rats treated with EEt(OH) *P.a* (5000 mg/kg b.w.) (8.22× 10^3/ml) on day 14 after the administration. These differences, however, were statistically significant (p<0.05). Similarly, the platelet count was slightly lower for the control rats (798.33× 10^3/ml) compared to the rats treated with EEt(OH) *P.a* (821.33× 10^3/ml). These differences, however, were statistically significant.

Action of the extract on the biochemical parameters explored

The various biochemical parameters explored, Glucose, Bilirubin (BIL), Alanine amino transferase (ALT), Aspartate amino transferase (AST), Total protein (TP), alkaline phosphatase (ALP), urea (URE), creatinine (CR), Total bilirubin (TBIL) and Indirect bilirubin (IBIL), have informed us about the probable effects of the stem leaves of *Phyllanthus amarus* in the liver and kidney. There was no significant differences (p>0.05) in glucose, total protein, urea, creatinine levels between control and experiemental groups at 14 d. Total bilirubin (TBIL) and Indirect bilirubin (IBIL), however, were statistically significant (p<0.05). Other biochemical parameters such as ALT, AST, ALP show statistically insignificant difference (p>0.05) in test and control batch.

DISCUSSION

This study examined a medicinal plant that is commonly

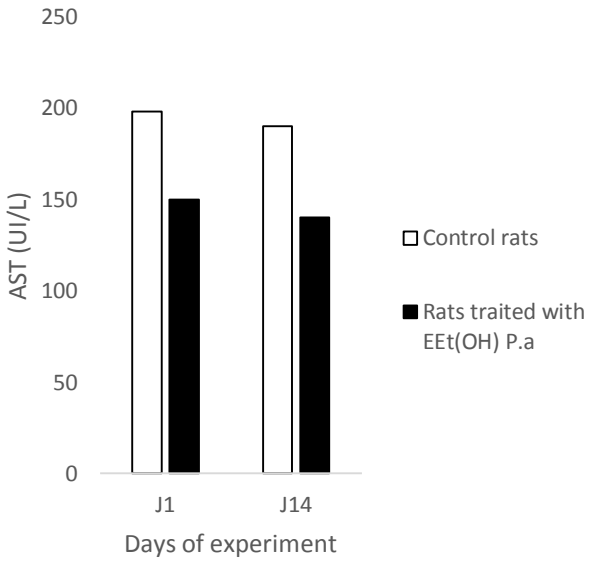

Figure 4. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on the AST transaminase, n=6, results represent the means±SEM (standard error of mean).

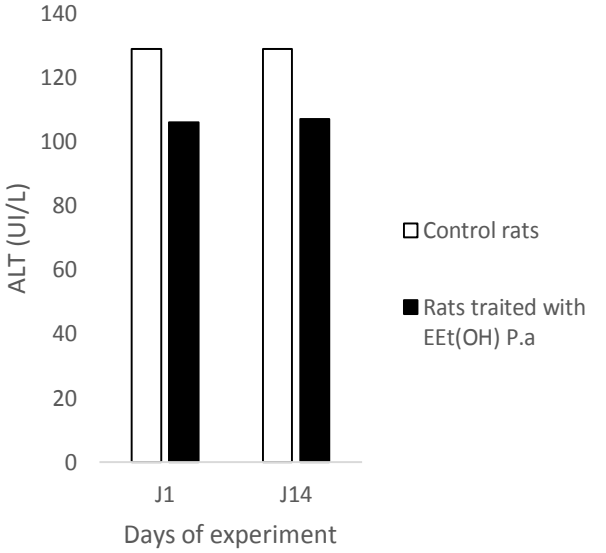

Figure 5. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on the ALT transaminase, n=6, results represent the means±SEM (standard error of mean).

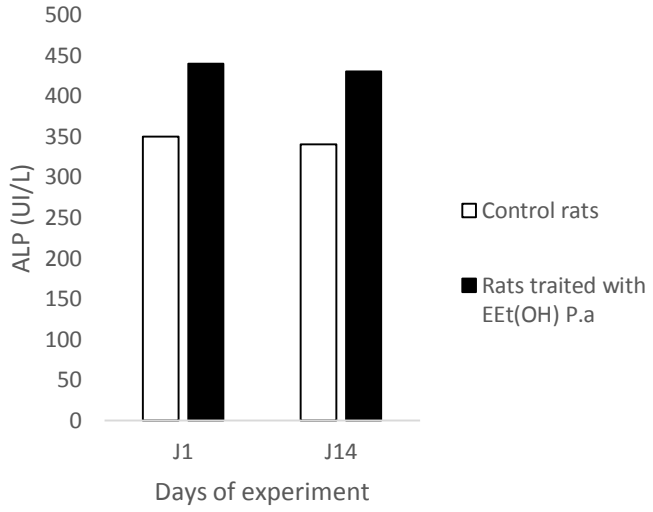

Figure 6. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on the alkaline phosphatases (ALP), n=6, results represent the means±SEM (standard error of mean).

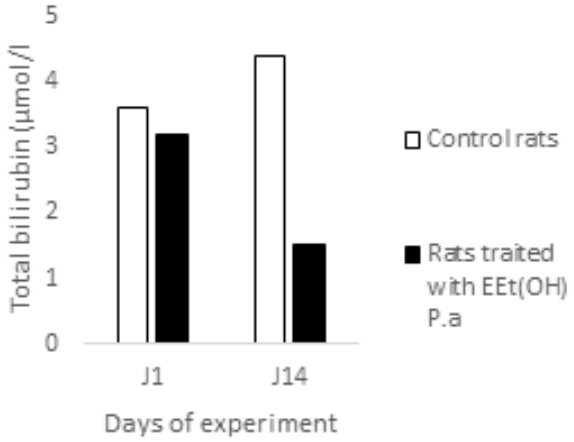

Figure 7. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on total bilirubin, n=6, results represent the means±SEM (standard error of mean).

used in the practice of traditional medicine (Patel et al., 2011) to determine possible safety risks. An animal model was used to identify effects on body weight, hematological indices and clinical chemistry. Such studies assess the hazard, namely the basic toxicity of the substance, and the risk is determined by considering the probability of exposure to a particular hazard at certain levels (Klaassen and Eaton, 1991).

Acute toxicity tests provide preliminary information on the toxic nature of a material for which no other toxicological information is available. Such information can be used to: (i) deal with cases of accidental ingestion of a large amount of the material; (ii) determine possible target organs that should be scrutinized and/or special tests that should be conducted in repeated-dose toxicity tests; and (iii) select doses for short-term and sub-chronic toxicity tests when no other toxicology information is available (Gad and Chengelis, 1988). Furthermore, the majority of pharmaceutical companies only use acute toxicity studies to determine the minimum lethal or maximum non-lethal dose. Thus, the aqueous leaf extract of *P. niruri* can be considered non toxic at the acute level and consequently, the lethal dose 50% (LD50) of *P.*

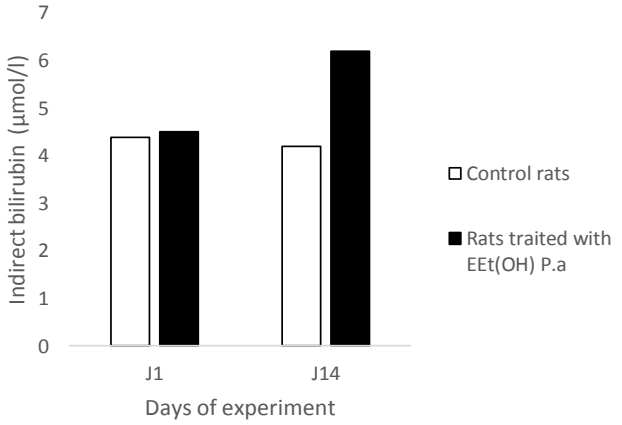

Figure 8. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on indirect bilirubin, n=6, results represent the means±SEM (standard error of mean).

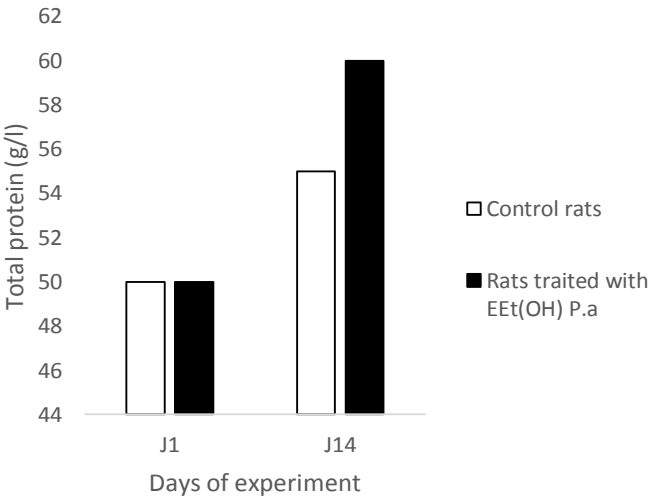

Figure 10. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on total protein, n=6, results represent the means±SEM (standard error of mean).

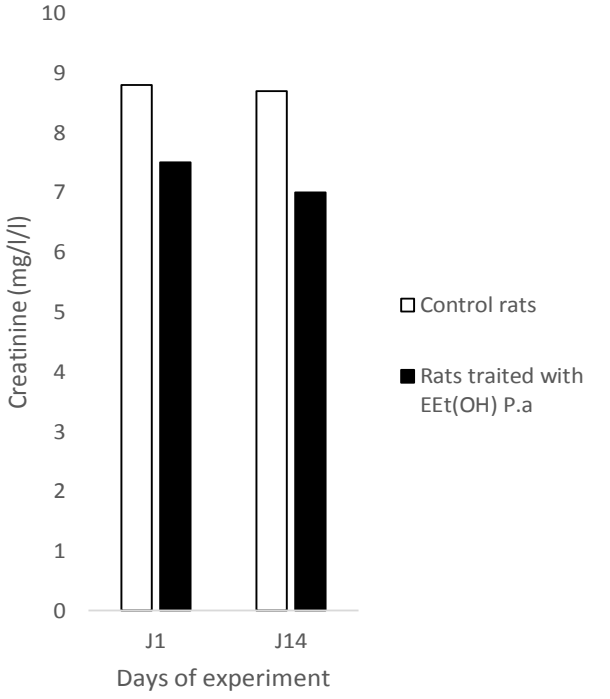

Figure 9. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on creatinine, n=6, results represent the means±SEM (standard error of mean).

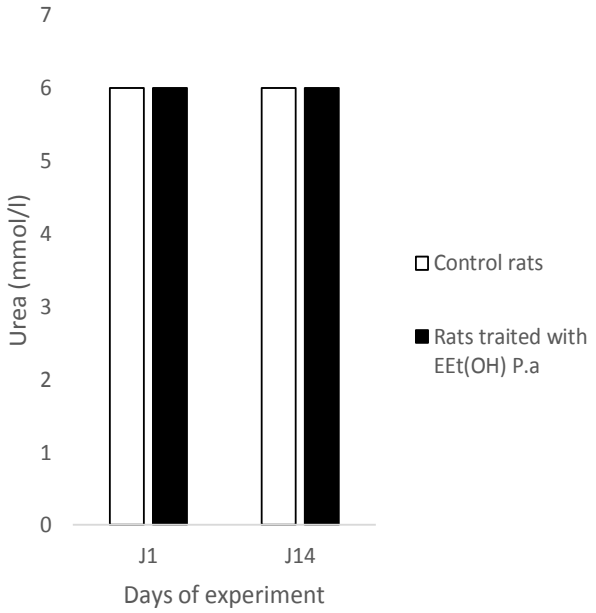

Figure 11. Effect of EEt(OH) *P.a* (5000 mg/kg b.w.) on urea, n=6, results represent the means±SEM (standard error of mean).

amarus Schumach. & Thonn. ethanolic extract is more than 5 000 mg/kg b. wt**.**

Analysis of the variation in body weight at the level of the batch having received EEt(OH) P.a and the control group showed a slight weight drop 7 days after treatment (Figure 1). This variation is not significant ($p>0.05$) and could be caused by other factors such as stress. The EEt(OH) P.a, therefore, has no effect on the weight change of the animals. Alternatively, previous work has shown (Butler, 1992; Carbonaro et al., 2001) 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 are comparable to the work (Guirou 2008) in the study of the sub-chronic toxicity of *Argemone mexicana*. Additionally, variation in body weight has been used as an indicator of the adverse effects of chemical compounds (Hilaly et al.,

2004). This loss of weight can be explained by a reduction consumption, or by the possibility of dose/absorption interactions and the reduction of the quantity of food absorbed. Other studies have also shown the weight reduction of rats after oral administration of the *Chicococca alba* extract (Gazda et al., 2006) and *Stryphnodendron adstringens* (Rebecca et al., 2002).

Organ body weight ratio is a useful index of swelling, atrophy or hypertrophy (Amresh et al., 2008). The absence of toxidromes was evident at the time of extract administration and thereafter. The biochemical data, mainly the hepatobiliary and renal systems, did not suggest any toxicity.

Assessment of haematological parameters can be used to determine the extent of foreign compounds including plant extracts on an animal's blood constituents. Such toxicity testing is relevant to risk evaluation as changes in haematological system have a higher predictive value for human toxicity, when data are translated from animal studies (Olson et al., 2000). It is can also be used to explain blood relating function of chemical compounds/plant extracts (Yakubu et al., 2007).

It is important to note that except for the 14 days blood WBC and platelet levels in treated group, all the hematological parameters present an insignificant statistical difference compared to the control group. The absence of the hematocrit variation leads us to exclude the hypothesis of hemoconcentration. The variations in the WBC could imply the condition. Thus, the ethanolic extracts of stem leaves of *P. amarus Schumach. & Thonn*. can be considered nontoxic at the acute level and consequently, the EEt(OH) *P.a* (5000 mg/kg b.w.) had no effect on plasma biochemical parameters.

With regard to the results obtained, we can deduce that the EEt(OH) *P.a* stem leaves 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 shows practically the safety of this plant used in the treatment of hepatitis by some traditional healers and herbalists in Benin. Serum enzymes AST, ALT are enzymes synthesized in the cytoplasm of the cell and discharged into the circulation in the case of damaged cells (Ozturk et al., 2009).

These are considered good indicators of hepatic cytolysis. Thus, high levels of liver enzymes, including ALT and AST, are frequently attributed to the metabolic and/or toxic effects of different drugs such as psychotropic drugs (Himmerich et al., 2005). At 5000 mg/kg body weight, AST and ALT did not increase. The realization of the histological sections will allow us to confirm these observations.

These results are comparable to those obtained by Guirou (2008) with the *Argemone mexicana* L., where they did not notice the variation of biochemical parameters during sub-chronic toxicity. This shows the safety of the ethanolic extract of this plant.

Direct and indirect bilirubin (rats treated with EEt(OH) *P.a* (5000 mg/kg) whose statistical difference compared to the control was significant ($p<0.05$). Administration of ethanolic extract of *P. amarus Schumach. & Thonn* (EEt(OH) *P.a*) after 14 days caused a marked significant ($p<0.05$) decrease in the total bilirubin levels. The result equally showed significant ($P<0.05$) increase in conjugated bilirubin level especially with the 5000 mg/kg dose which may be an indication of the safest dose to use when administering this extract for medicinal purpose. Evaluation of proteins are good criteria for assessing the secretory ability/functional capacity of the liver (Naganna, 1989). The non-significant effect of the extract on total protein in the serum of animal at the dose investigated could imply that the synthetic and secretory functions of the liver with respect to these proteins were not affected.

Liver protective herbal drugs contain a variety of chemical constituents like coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavonoids, organic acids, lipids, alkaloids, xanthenes, terpenoids, reducing sugars, resins saponins and stilbeans (Gupta et al., 2006). These constituents also are present in *P. amarus Schumach. & Thonn*. It has been suggested, based on *anecdotal* evidence that the stem leaves extract of *P. amarus schumach & Thonn*. could prevent damage to the liver.

In order to mitigate or eliminate their toxicity and the secondary effects of these drugs, a plethora of certain techniques have been used involving drug treatment and compatibility. Some plants containing aristolochic acids such as *A. manushuresis* (Hu et al., 2004; Wang et al., 2005), *A. fangchi* (Hu et al., 2003; Yang et al., 2011) and *A. radix* (Wang et al., 2007) have been reported to reduce toxicity (Wang et al., 2007; Li et al., 2013; Hu et al., 2004; Yang et al., 2011).

Conclusion

The ethanolic extract of the stem leaves of *P. amarus Schumach. & Thonn*. (EEt(OH)P.a) on this limited sample of animals did not have a toxic effect on the biochemical and hematological parameters studied at a dose of 5 000 mg/kg. b.w. The lethal dose is therefore over 5 000 mg/kg. b.w. Determination of the alanine aminotranferase content and aspartate aminotransferase content on the rats which received extract showed no variation when compared to the panel of control. 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. Carrying out of the exploratory tests of cytotoxicity are imperative to determine any possible secondary effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

EEt(OH)P.a, Ethanolic extract of the stem leaves of *Phyllanthus amarus Schumach. & Thonn*.; **WBC,** white blood cells; **AST,** aspartate amino transferase; **ALT,** Alanine amino transferase; **ALP,** alkaline phosphatase; **BIL,** bilirubin; **TBIL,** total bilirubin; **IBIL,** indirect bilirubin; **TP,** total protein; **CR,** creatinine; **URE,** urea.

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