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Full Length Research Paper

Toxicological assessment on extracts of *Phyllanthus amarus* Schum and Thonn

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Phyllanthus amarus **is used in traditional medicine to treat diabetes. The purpose of this study is to evaluate its toxicity. The cytotoxicity of its aqueous extracts (AE) and hydroalcoholic extracts (HAE) on Caco-2 cells was evaluated using neutral red uptake and MTT test. Aqueous extract was more cytotoxic than hydroalcoholic extract (IC50 being 89.6 µg/ml versus 277 µg/ml). Acute and sub acute toxicity of the extracts were evaluated respectively in Swiss mice and Wistar rats. A single oral dose of the extracts at 5 g/kg b.w. did not produce mortality or any significant change in treated animals over a 14 day observation period. In the sub acute toxicity study, extracts were administrated (1 and 3 g/kg b.w.) daily by gavage to rats for 28 days. No significant differences were observed in body weight gain and blood glucose levels between controls and treated groups. Clinical biochemistry revealed no toxic effect. Neither gross abnormalities nor histopathological changes in liver, kidney and pancreas were observed. Extracts of *P. amarus* could then be considered to be safe in animals by oral route (LD 50 > 5 g/kg) even though it is slightly cytotoxic to the human adenocarcinoma cell line Caco-2.**

Key words: Caco-2 cells, *in vitro* toxicity, *Phyllanthus amarus*, rat, sub acute toxicity.

INTRODUCTION

Phyllanthus amarus Schum and Thonn (Euphorbiaceae) is an herbal plant which widely spread throughout the tropical and subtropical areas. It is one of the most important medicinal plants used in traditional medicine for the treatment of diabetes and excessive body weight reducetion. Extracts of the plant had been reported to have pharmacological effects such as antibacterial (Mazumder et al., 2006; Kloucek et al., 2005), antiviral activity against hepatitis B (Thamlikitkul et al., 1991; Yeh et al., 1993; Munshi et al., 1993). *P. amarus* has also been used as chemoprotective agent (Kumar and Kuttan, 2005), antimutagenic (Raphael et al., 2002a; Sripanidkulchai et al., 2002) and exhibits hypoglycaemic properties (Lawson-Evi et al., 1997; Srividya and Perival, 1995; Raphael et al., 2002b). Aqueous extract of *P. amarus* bears antispasmodic properties (Gbeassor et al., 1988). Several active compounds have been identified in *P.* phyllanthin and hypophyllanthin (Somanabadhu et al., 1993), flavonoïds such as quercetin and astragalin (Nara et al., 1977 and securinega-type alkaloids such as isobub-bialine and epibubbialine (Houghton et al., 1996). Gallo-tannin and ellagitannins (geraniin and corilagin) were shown to be the most potent mediators of the antiviral HIV activity (Notka et al., 2003). Hydrolysable tannins purified from *P. amarus* were found to be potent inhibitors of rat liver cyclic AMP-dependent protein kinases (Polya et al., 1995). Phyllanthin and hypo-phyllanthin present in *P. amarus* are reported to be hepatoprotective agents and protect hepatocytes against carbon tetrachloride and galactosamine-induced liver toxicity in rats (Syamasundar et al., 1985).

In Togo, *P. amarus* is found easily during the rainy season and is consumed by the population as preventive mean against all kind of diseases.

Early studies in our laboratory confirmed that extracts of *P. amarus* harvested in Togo are rich in tannins and phenols but poor in alkaloids and this is in line with previously published data (Polya et al., 1995; Houghton et al., 1996). Due to the worldwide usage of *P. amarus* in traditional medicine, this study was carried out to assess the toxicity of water and hydroalcoholic extracts in Caco-2 cells (a human colon cancer cell-line), on Swiss mice and

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Wistar rats, with the purpose that the results would provide information on the safe use of this plant.

MATERIALS AND METHODS

Plant material

The whole plant of *P. amarus* was collected from Tsevie (at 36 km North-East of Lome, Togo). A voucher specimen was identified and kept in the herbarium of the Laboratory of Botany and Plant Ecology (Faculty of Science/University of Lome) under the reference No 3547. Plant was cleaned out with water and cut into small pieces, dried and extracted with water or ethanol/ water (1:1 v/v) as following. Plant material (200 g) was soaked in water (2 L) and heated in a water bath at 80°C for 1 h for the aqueous extract. The same quantity was macerated 72 h in ethanol/water solution (2 L) for hydroalcoholic extract. The crude extracts were filtered with Whatman paper and evaporated in vacuo at 40°C using a rotary evaporator. The yield of the preparation was 16% (w/w) for aqueous extract (AE) and 13% (w/w) for hydroalcoholic extract (HAE).

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS), Phosphate-Buffered Saline (PBS), trypsine-0.02% EDTA mixture, MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich (Lyon, France). All other chemicals used were of analytical grade and from Sigma chemicals, France.

Cell culture

Caco-2 cells, originating from a human colon cancer were obtained from Dr Jing Yu, (Tuffs School of medicine, Medford, Mass, USA). Cells were routinely cultured at 37°C in a humidified 5% CO_2, 95% air atmosphere. Cells were grown in DMEM medium (Sigma, France) supplemented with 10% foetal bovine serum, L-glutamine (8 mM), penicillin (100 UI/ml), and streptomycin (100 µg/ml).

Neutral red uptake assay

Cells were seeded in 96 well microplates (10000 cells/ 200 µl) and cultured in a humidified incubator for 24 h. They were maintained in culture and exposed to extracts of *P amarus* over a range of concentrations (1 - 1000 µg/ml). After 72 h exposure to extracts, neutral red uptake test (NR) was performed according to procedure of Yusup et al. (2005). Briefly culture medium was discarded and 200 µl of freshly prepared neutral red in DMEM (50 µg/ml) were added to every well. Cells were then re-incubated at 37°C for an additional 4 h. After this period of incubation, cells were carefully washed twice with 200 µl of PBS to eliminate extra-cellular neutral red. The incorporated dye was eluted from the cells by adding 200 µl of elution medium (50% ethanol supplemented with 1% acetic acid, v/v) to each well. Microplates were shaken gently for 10 min and read at 540 nm using microplates reader (Dynatech. MR 4000, Boston, MA, USA).

MTT assay

Cells were seeded in 96-well microplates (10000 cells/200 µl/well) and cultured in a humidified incubator for 24 h. After this period of incubation, cell culture medium was removed and replaced by medium containing extracts except control wells, at concentrations ranging from 1 to 1000 µg/ml for 72 h. At the end of this period, medium with or without herbal extract was discarded and 100 µl of MTT solution (0.5 mg/ml in DMEM) was added to every well. And 2 h later, 100 µl of DMSO solution were added to each well to dissolve the formed formazan crystals. Microplates were shaken gently for 10 min and read at 540 nm using a microplate reader (Dynatech MR 4000, Boston, MA, USA). Four wells were used for each extract concentration.

Animals

Male and female Swiss mice weighing 22 - 23 g were obtained from the animal centre R. Janvier (France). Wistar rats (120 - 150 g) were purchased from animal centre DEPRE (France). Animals were housed under standard environmental conditions with free access to conventional chow (toxins-free according to EU regulation) and tap water ad libitum. They were deprived of food except water 10 - 16 h prior to the experiments.

Acute oral toxicity assay in mice (Limit test OECD 401)

The acute oral toxicity study was conducted using the limit test procedure according to OECD Test Guidelines on acute oral toxicity (401). This test was conducted on mice. Four groups of 6 animals (3 males and 3 females) were selected; animals received by single gastric intubations either 2 g or 5 g/kg of extracts (AE, HAE) dissolved in distilled water and were observed for mortality, signs of gross toxicity or behavioural changes (excitability, convulsions, lethargy, sleep) one hour post dosing and at least once daily for 14 days.

Sub acute toxicity in rats (OECD 407)

Six groups of 10 animals (5 females and 5 males) were used. Rats were treated daily for 28 consecutive days. The test groups received orally by gavage the extracts dissolved in distilled water at the doses of 1 g/kg and 3 g/kg. Distilled water was given to the controls (one group control for each extract). During the experimental period, each animal was observed daily for signs of toxicity. Blood glucose levels and body weight changes were monitored weekly. Twenty four hours after the last dose, animals were anaesthetized with ether. Blood was collected from the retro orbital sinus to yield serum used for blood analysis. Animals were killed by cervical dislocation prior organs collection. Pancreas, liver and kidney were collected from each animal for morphological analysis.

Blood glucose measurement

Blood was taken on tail vein of rats and measured with a glucometer (Optium Sense).

Biochemical measurements

Blood samples collected by the orbital sinus puncture were centrifuged at 500 x g and serums were kept at –20°C. The activities of aspartate aminotransferase, EC. 2.6.1.1 (ASAT), alanine aminotransferase EC 2.6.1.2 (ALAT), γ-glutamyltransferase, EC 2.3.2.2 (GGT), creatine phospho-kinase, EC 2.7.3.2 (CK), creatinine (CREAT), were measured using standardized commercially availa-

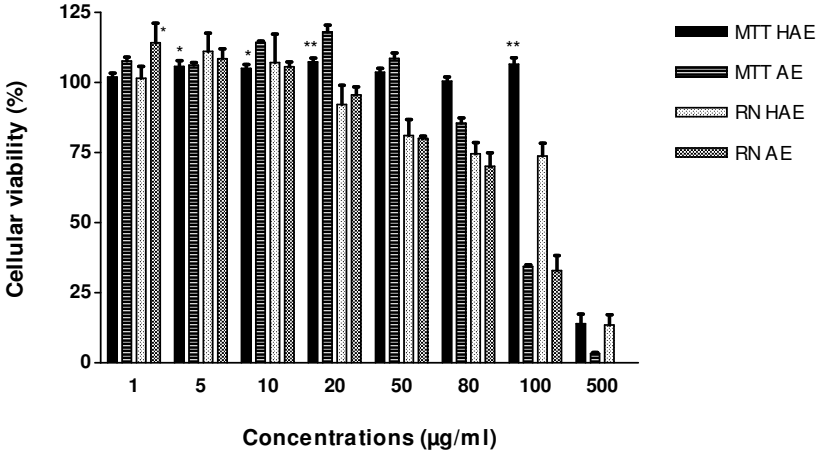

Figure 1. Effect of aqueous extract (AE) and hydroalcoholic extract (HAE) on cellular viability (%). Each point represents mean ± SEM. * $P < 0.05$; ** $P < 0.01$ vs 100% viability (considered as controls).

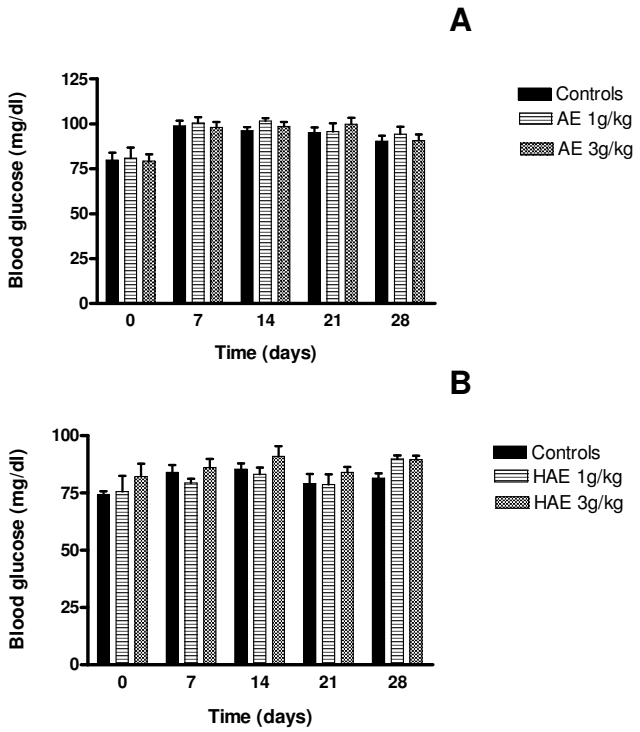

Figure 2. Effect of aqueous extract AE (**A**) and hydroalcoholic extract HAE (**B**) on blood glucose level after 28 days administration. Blood glucose levels were measured before the administration of the extract on day 0 and on days 7, 14, 21 and 28. Each point represents mean ± SEM of 6 rats.

Urinalysis

Prior to the completion of the administration period (day 28), the animals were placed individually in metabolic cages and urine was collected for 8 h. The collected urine was tested for creatinine andble kit (BioMérieux).

Urinalysis

Prior to the completion of the administration period (day 28), the animals were placed individually in metabolic cages and urine was collected for 8 h. The collected urine was tested for creatinine and xcommercially available kit (BioMérieux).glucose level using standardized commercially available kit (BioMérieux) and glucose sticks test papers (glucotest) respectively.

Histopathological studies

Excised liver, pancreas and kidney were preserved in 10% buffered formaldehyde solution for histopathological examination.

Statistical analysis

All data were expressed as means ± SEM. 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 $P < 0.05$. Figures were performed using Graph Pad Prism 4.

RESULTS

Evaluation of cell viability

Cells viability was evaluated using neutral red and MTT test. Aqueous extract of *P. amarus* was more effective in reducing cell viability than hydroalcoholic extract. At low concentrations, extracts (aqueous and hydroalcoholic) exhibited a proliferative effect in Caco-2 cells (Figure 1).

Acute oral toxicity assay in mice

No signs of toxicity, behavioural changes or mortality were observed in the course of this test during two weeks of observation.

Table 1. Body weight gain (g) after 28 days of treatment with aqueous extract (AE).

Sex	Control	AE (1 g/kg)	AE (3 g/kg)
Females	77 ± 4.14	66 ± 5.05	64.6 ± 5.88
Males	126.8 ± 6.01	131.6 ± 6.13	141.6 ± 4.83

*Body weight gain (g) is expressed as difference between final and initial body weight.
Each point represents mean ± SEM of 10 rats.

Table 2. Body weight gain (g) after 28 days treatment with hydroalcoholic extract (HAE).

Sex	Control	HAE (1 g/kg)	HAE (3 g/kg)
Females	68 ± 8.89	85 ± 4.43	69 ± 6.90
Males	116.6 ± 7.18	123.8 ± 7.60	104.2 ± 6.27

*Body weight gain (g) is expressed as difference between final and initial body weight.
Each point represents mean ± SEM of 10 rats.

Sub acute toxicity in rats

Body weight gain after 28 days of treatment in the three groups was not statistically different although a slight decrease was observed in females receiving aqueous extract of *P. amarus* (Table 1). Rats treated with HAE showed no decrease in body weight gain compared to the control group (Table 2). During the 4 weeks treatment, the extracts had no significant effect on blood glucose level. The values of blood glucose are similar in the three groups (Figure 2).

Effect on blood biochemistry and urinalysis

Serum biochemical data and urinalysis for aqueous and hydroalcoholic extracts are presented in Tables 3 and 4 respectively. No significant changes were observed in the biochemical parameters (ASAT, ALAT, γ-GT, creatinine, CK) between treated groups and control. There was also no difference observed between treated groups and control in respect of urinary parameters such as glucose and creatinine.

Histopathological studies

The histopathological examination of liver, kidney and pancreas excised from these rats showed no pathological changes (data not shown).

DISCUSSION

In vitro, aqueous extract of *P. amarus* was more cytotoxic to Caco-2 cells than hydroalcoholic extract. This effect was concentration dependent and suggested that there are small differences in the composition of the two extracts. At low concentrations, AE and HAE induced cells proliferation. This could be due to antioxidant substances present in the extracts which are known to display biphasic properties, being antioxidant at low concentration and pro oxidant at high concentration. This antioxidant activity may help preventing oxidative stress and lipid peroxidation favouring somehow cell proliferation (Young et al., 2007; Català 2007). This is indeed confirmed here with decreased of Caco-2 cells viability *in vitro* at concentration above 80 µg/ml for AE or 100 µg/ml for HAE. Low concentrations led to 10 - 15% of cell proliferation.

At a dose of 5 g/kg, mice did not show any sign of toxicity and no mortality was observed. In the present study, administration of AE or HAE during 28 days treatment did not reveal any significant decrease in body weight gain compared to control, except in females for which a slight but not statistically significant weight loss was observed for animals treated with the aqueous extract. This could be explained by the fact that the AEs contain much more tannins than the HAEs. Tannins are known to display antinutritional properties (Butler, 1992; Carbonaro et al., 2001).

The extracts have no effect on fasted blood glucose level. This result confirmed our previous study on *P. amarus* in albino rabbit (Lawson-Evi et al., 1997).

Liver is the primary site of biotransformation and detoxication of xenobiotics. Thus the liver is especially vulnerable to xenobiotics (Lee, 1993; Sturgill and Lambert, 1997). Similarly, kidneys as the principal organ for the excretion of xenobiotics and their metabolites are particularly prone to their toxic effects (Subcommittee on Biologic Markers in Urinary Toxicology, 2002). Damage to these organs often results in elevation in clinical biochemistry parameters such as serum enzymes; ASAT, ALAT and GGT and/or creatinine as a marker of impairment of renal filtration (Ferguson et al., 2008). We did not observe any abnormality in serum chemistry parameters and in urinalysis that would suggest that treatment with hydroalcoholic or aqueous extract of *P. amarus* has any adverse effect on either liver or kidneys. γ-Glutamyltransferase level was significantly low ($P < 0.05$) in the group receiving aqueous extract of *P. amarus* at 3 g/kg, but this level falls within normal range (Derelanko, 2000). Values of creatine phosphokinase (CK) were in normal range indicating that the extracts neither caused damage to cardiac or skeletal muscles. However Adedapo et al. (2005a) had reported that some chromatographic fractions obtained from *P. amarus* had potentially deleterious effects on the blood biochemistry. They also showed that, aqueous extract of *P. amarus* leaves induced some pathological changes in rat necrotic foci with lymphocytes infiltration in the liver, seminiferous tubules devoid of spermatic cells following 30 days

Table 3. Effect of aqueous extract (AE) on serum biochemical parameters after 28 days treatment.

Parameter	Control	AE (1 g/kg)	AE (3 g/kg)
Serum			
ASAT (UI/l)	68.01 ± 4.67	62.21 ± 4.01	67.21 ± 4.18
ALAT (UI/l)	21.02 ± 2.2	17.7 ± 0.78	21.42 ± 1.7
GGT (UI/l)	0.87 ± 0.18	0.53 ± 0.07	0.48 ± 0.1*
CREAT (µmol/l)	39.78 ± 1.56	42 ± 1.56	44.93 ± 0.97
CK (UI/l)	262.28 ± 21.8	264.57 ± 48.64	290.7 ± 39.35
Urine			
CREAT (µmol/l)	4 ± 0.6	2.11 ± 0.09	4.5 ± 0.75

ASAT = Aspartate aminotransferase; ALAT = alanine aminotransferase; GGT = γ-glutamyl transpeptidase; CREAT = creatinine; and CK = creatine kinase.
Data are means ± SEM of 6 rats (male and female); * $P < 0.05$ vs control.

Table 4. Effect of hydroalcoholic extract (HAE) on serum biochemical parameters after 28 days treatment.

Parameters	Controls	HAE (1 g/kg)	HAE (3 g/kg)
Serum			
ASAT (UI/l)	78.77 ± 2.32	72.05 ± 6.7	82.1 ± 5.98
ALAT (UI/l)	13.68 ± 0.64	12.36 ± 0.62	16.82 ± 2.16
GGT (UI/l)	0.75 ± 0.16	1.19 ± 0.2	0.94 ± 0.23
CREAT (µmol/l)	43.09 ± 1.48	43.09 ± 2.83	48.62 ± 2.21
CK (UI/l)	129.54 ± 9.68	164.12 ± 22.22	140.77 ± 20
Urine			
CREAT (µmol/l)	4.13 ± 0.98	3.46 ± 0.46	3.74 ± 0.37

ASAT = Aspartate aminotransferase; ALAT = alanine aminotransferase; GGT = γ-glutamyl transpeptidase; CREAT = creatinine; and CK = creatine kinase.
Data are means ± SEM of 6 rats (male and female).

treatment with *P. amarus* (Adedapo et al., 2005b). These differences with our results may be explained by the specification of the herbal samples and/or strain of rat. Moreover, no other data could be found in the literature reporting such a drastic effect on male reproductive organs.

The extracts of *P amarus* samples could be classified as non toxic having LD50 > 5 g/kg bw according to the OECD regulation (OECD 417). To our knowledge this is the first report on the lack of toxicity of *P. amarus* for which pharmacological and therapeutic properties are well documented (Moshi et al., 2001; Srividya and Perival, 1995).

Due to the large scale use of *P. amarus* in Africa as well as in Asia, toxicological data are needed. These data may be used to help preparing traditional improved medicine based on these extracts as it is being currently done following the recommendation of WHO (2000).

In conclusion, *P. amarus* extracts are found to be non-toxic in mice and rats following either a single dose or daily repeated doses for 28 days; but for final safety, assessment of genotoxicity, mutagenesis and reproduction toxicity will be needed.

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