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FREE-RADICAL SCAVENGING ACTIVITY AND TOXICITY STUDY OF PSILOTUM NUDUM (L.) P. BEAUV., A LOWER PLANT ACCLIMATED IN NORTH BENIN

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ABSTRACT
Both of higher and lower plants provide healthcare source for the population in rural and urban area in Benin and in the world. While some are explored for their biological properties, others remain less known. The aim of this study is to assess the phytochemical and toxicity properties of *Psilotum nudum* in rat. We used in vitro complexion and/ or precipitation reactions to detect the presence or absence of different groups of secondary metabolites in that Pteridophyte. The antiradical scavenging activity is shown by two different radical : DPPH and Hydroxyle radical. Blood biochemicals analysis before and after force-feeding (100 mg/kg of Body Weight (BW), 1000 mg/kg BW and 2500 mg/kg BW) and physical observation were used for toxicity assessement. Psilotum nudum contains different phytochemical compounds such as tanins and flavonoids likely responsible of the extract free radical scavenging activity. Changes observed in blood biochemical parameters [Urea, Creatinine, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT)] after seven days of extract administration normalised after 30 days of observation.
Upto this stage of our research, aqueous extract of P. nudum should be taken with caution.

KEYWORDS: *Psilotum nudum* -scavenging activity-toxicity-Benin.

INTRODUCTION
The use of plants for healthcare and medical purposes is for several thousands of years. Both higher plant species and lower ones are used and all vegetable organs are of interest, (leaves, stem bark, roots, fruits, flowers) for treating several infectious diseases such as fever, headache, wounds, diarrhea, dysentery, cholera, malaria and other diseases.[1] Herbal medicines remain one of the source to which populations in developping countries refer for their health needs and provide first-line and basic health service due to it availability, affordability to the to people living in poor areas.[2] Plants are used for both treatment and preventive measures in disease control strategies.[3] Their nutraceuticals source purpose gains more importance in order to promote health and nutrition. Even though Angiosperms are well known for their therapeutic importance, lower plants gained particular attention in recent year in the purpose of new and effective molecules discovery. Among those lower plants, pteridophytes to which genus Psilotum belong, are well known of traditional medicine for long time.[4] The genus Psilotum consists of two species, P. nudum (L.) P. Beauv. (= P. triquetrum Sw) and P. flaccidum Hook. & Grev. (= P. complanatum Sw.) and is characterized by dichotomously branched stems, the absence of roots, a relatively simple vascul ar structure, small reduced leaves and thick-walled, homosporous synangia.[5] In Benin, Psilotum nudum is met in north part of the country (Assotè, Sèmèrè, Bétérou) having a tropical climat[6] where the entire plant is used against malaria and fatigue and as antianemic.

Psilotum nudum (L.) P. Beauv is an epiphyte that grows as a terrestrial plant in rocky crevices or in sandy soils and being a spore-producing vascular plant is considered to be a fern ally. Ethnomedicinal uses of P. nudum are as general pain reliever especially dental pains and for easing bowls.[4,6] Assessing its properties, Rani et al (2010) found that chloroformic, ethanolic and aqueous

extracts of P. nudum possess fungal and bacterial properties.[4]

Green tea polyphenols are the secondary metabolites in tea plants acting as antioxydants in the biological system by neutralizing free radicals and may reduce or even help to prevent some of the damage caused by reactive oxygen species (ROS).[7]

In order to ensure of their use safety and effectiveness, plants of traditional medecine should undergo, phytochemical, toxicological and pharmacological trials since their traditional and popular use do not guarantee the safety and efficacy of medicinal preparations.[8]

Thus, this work study is designed to carry out the phytochemical screening, the in vitro anti-oxidant activiy and the subacute toxicity of aerial part of P. nudum.

MATERIAL AND METHODS

Plant material

Vegetable material is constituted by aerial part of P. nudum collected at Ouake (Donga, Benin) in dried tropical climat zone, in June 2016 and identified in National herbarium (Université d'Abomey-Calavi). The collected material are dried in laboratory temperature conditions. It has then been reduced in powder that constitute our sample to be analyzed and from which we obtained the crude extract for further use.

Animals

Male and female Wistar strain albino rats (160 to 200g) of eight weeks old reproduced in our animal house (Laboratory of Animal Physiology and experimental pharmacology, FAST/UAC) have been used. They were maintained under standard laboratory conditions (12 h light and dark cycle) had free access to standard chow and drinking water. We accustomed them to the experimental conditions prior to the experiment (handling, blood sampling, labelling by permanent marker pen…) to avoid stress-induced variation in biochemical parameters.

Preparation of the extract

Per plant material section, we prepared crude extract from material powder obtained. For this purpose, the powdered material (200g) was macerated in 1L of distilled water and filtered after 24 h. Residues are then remacerated for another 24 hours, filtrated and once again remacerated for one more 24 hours and filtrated. All filtrates were ketp and evaporated in an oven set at 40° C until (two weeks) a constant weight was obtained. Three different bottles were used. The dried extract was weighted, packed and stored in dried place.

The percentage yield (P) of the extract was calculated using the expression:

$$P = \left(\frac{Wext}{Wprd}\right) * 100$$

With :

Wext = weight of the dried concentrated extract.
Wprd = weight of the powdered leaves.

Phytochemical screening

It consists in identifying for a plant, chemical compounds groups showing pharmacological/toxicological interest. The powder was used for that qualitative analysis. The qualitative analysis is based on precipitation and coloration reactions as described by Houghton and Raman (1998).[9] Some secondary metabolites have been essayed such as Alkaloids (Mayer's test), Cathetic tannins (stiasny test), Gallic tannins (ferric chloride test after saturation with sodium acetate), Flavonoids (shinoda test and magnesium powder), Cyanogenic derivatives (picric acid test), Saponins (test index foam), Mucilage (test of absolute alcohol), Coumarins (test with ether and ammonia), Free anthracene derivatives (test with chloroform and ammonia).

Evaluation of the radical-scavenging activity of AEPN

DPPH radical scavenging assay

The principle of this method is based on measuring the trapping of free radicals by DPPH solution. This trap is shown by the disappearance of the purple color of DPPH. Mixing of reagent (4% of DPPH in ethanol) and extract are left in the dark for an hour and the absorbance was measured at 517 nm.[10] Assays are perfomed triplicate. The percentage of scavenge was determined by the formula:

$$P = ((Ab-Ae)) / Ab \times 100$$

With P: Scavenge percentage; Ab: absorbance of the blank, Ae: Sample Absorbance.
The 50% of the radical Inhibiting Concentration (IC_{50}) is determined graphically as shown by the Figure 1

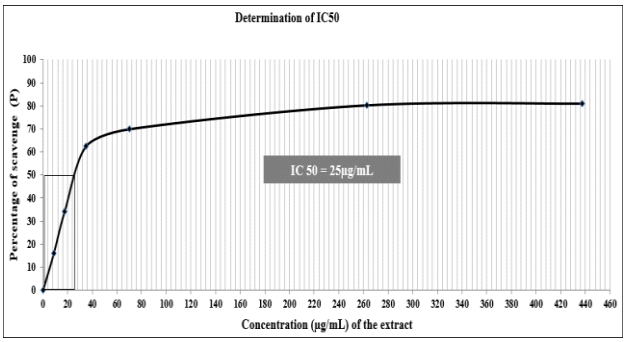

Figure 1: IC50 determination graph.

Hydroxyl radical scavenging assay

This test is performed according to the method described by Su et al (2009).[11] Briefly, 2 ml of the aqueous $FeSO_4$ solution (6 mM) were added to 2 ml of extract and 2 ml of H_2O_2 (6 mM) were added there to. After 10-min incubation at room temperature, 2 ml of an aqueous solution of salicylic acid (6 mM) were added to the mixture and the whole incubated again for 30 min. the absorbance is measured at 510 nm. The radical

scavenging activity was calculated as stated above. Assays are perfomed triplicate

Experimental design

A total of 20 rats was divided into four (4) groups after weight measurement to keep the batch homogeneity. Groups were named G0 (for control group receiving distilled water instead of drug), G1 (group receiving 100 mg/kg of body weight of drug), G2 (group receiving 1000 mg/kg of body weight) and G3 (group receiving 2500 mg/kg of body weight). All animals were kept fasting for overnight providing only water, after which the extracts were administered orally by 2mL of either distilled water or drug at different concentration.

Acute toxicity study

Acute toxicity was performed to determine the toxicity class of the plant. After drug administration, animals were observed for 6 hours and then general symptoms of toxicity (piloerection, convulsions, lethargy, diarrhea and sleep)[12] and mortality were recorded for 24 hours.

Sub-acute toxicity studies

After the 24 hours of observation three animals of each groups underwent six (06) more days of force feeding of the drug at the same concentration according to their group. They have also been kept observation for 30 days from the end of seven-day drug administration.

Blood was sample three times : Seven days before drug administation (D0), 14 days later (D+7) and at the end of observation period (D+30). Alanine aminotransferase (Alat), Aspartate aminotransferase (Asat), urea and creatinine have been estimated at each blood sampling using spectrophotometer KENZA MAX BioChemisTry PHOTOMETER according to standard procedures provided along with the kits supplied by biolabo (France).

Data analysis

Minitab 16. was employed and data analyzed using one way analysis of variance (ANOVA) followed by Turkey multiple comparison test. Values were considered significant at $p<0.05$.

RESULT AND DISCUSSION

Principal chemical groups identified in aqueous extract of P. nudum (AEPN) are consigned in the following table 1.

Table 1: Secondary metabolites in P. nudum.

		Psilotum nudum
Tanins	catechic	-
	gallic	+
Anthocyans		-
Leucoanthocyan		+
Anthraquinones		+
Alcaloides		+
Flavonoides		+
Mucilages		+
Saponosides		-
Terpens et sterols		+
Coumarines		-

Phytochemical study reveals the presence of gallic tannins, anthocyanins, leucoanthocyanins, alcaloids, flavonoids and Terpens and sterols. Compounds such as catechic tanins, anthocyans, saponoside and coumarines are absent in our sample. 5-Hydrixypsilotin and others molecules belonging to phelonic compound have been isoloted from P. nudum.[13] This pteridophyte is reported to contain an alkaloid psilotin, an insect feeding deterrent and growth reducer.[13] Moreover, Tannins possess antioxidant, antimicrobial, anti-inflammatory, antiviral, antifungal, anthelmintic and anti-tumour properties and Flavonoids have antioxidant and detoxification activities.[14] Both of the two compound groups are present in our sample, that could explain antifungal and antibacterial activities of P. nudum.[4]

4.25 ± 0.28 % is the percentage of yield of AEPN. The literature doesn't provide similar work about neither P. nudum nor P. flaccidum (the second specie of the genus Psilotum). That relatively low yield could be attributed to the lack of leaves.

Radical-scavenging activity of AEPN

The following table 2 presents the IC_{50} of Aqueous Extract of P. nudum (AEPN) using two different assays. We performed these essays not only on AEPN but also on two antioxidant reference compounds : Quercetin and Gallic acid.

Table 2: The IC 50 (µg/mL) of Aepn.

	IC_{50} (µg/mL)	
	DPPH radical	Hydroxyle radical
AEPN	27 ±2.50	18 ± 3.00
Quercetin	3 ±1.00	25 ± 1.5
Gallic acid	23 ±3.50	135 ± 6.75

The above table reveals that AEPN possess less anti-radical activity than the two pur reference antioxydants (Quercetin and Gallic acid) using DPPH radical. Howerver this IC_{50} of 27 could be biologically active. In comparison to pur reference antioxydants (Quercetin and Gallic acid) and using Hydroxyle radical, the AEPN shows higher anti-radical activity (18 versus 25 or 135).

Hydroxyle radical belong to free radicals found in organism and anti-radical substance (such as plant/fruit extract) could reduce the oxidative stress induced by free radicals by quenching the hydroxyl radicals that trap HO leading to oxidative breakdown of the carotenoids molecule.[15] Thus, in vivo anti-oxydant activity assessment of AEPN could be useful for the use of P. nudum earial stem as nutraceuticals source. Moreover, the antifungal antibarcterial activities of P.nudum[4] could be related to its anti-radical scavenging properties.

Animal blood biochemicals

The following table 3 presents rat's blood biochimestry parameters

Table 3: Blood biochemestry parameters.

	Dose	Control batch	G1 (100mg/Kg BW)	G2 (1000mg/Kg BW)	G3 (2500mg/Kg BW)
Urea (mg/ml)	D0	0.59 ± 0.06	0.55 ± 0.10	0.71 ± 0.14	0.67 ± 0.17
	D+7	0.56 ± 0.23	0.53 ± 0.08	0.47 ± 0.12 ¥	0.50 ± 0.05 ¥
	D+30	0.54 ±0.12	0.57 ± 0.10	0.67 ± 0.13	0.64 ± 0.15
Creatinin (mg/l)	D0	6.18 ± 0.19	6.52 ± 0.67	5.44 ± 0.55	6.21 ± 0.43
	D+7	7.20 ± 0.24	7.70 ± 0.54	8.02 ± 0.42 ¥	8.00 ± 0.35 ¥
	D+30	8.04 ± 0.45	7.03 ± 1.02	6.15 ± 0.85	7.18 ± 1.10
ASAT (U/dl)	D0	32.01 ±2.30	33.47 ± 4.36	35.84 ± 0.92	23.13 ± 1.61
	D+7	29.91 ± 2.12	23.14 ± 2.67 ¥ *	28.26 ± 2.06 ¥	25.82 ± 5.14
	D+30	33.24 ± 1.85	28.65 ± 2.15	31.45 ± 3.16	26.58 ±2.80
ALAT (U/dl)	D0	7. 96 ± 0.67	8.02 ± 0.74	8.80 ± 0.261	8.09 ± 0.55
	D+7	8.12 ± 0.15	6.91 ± 0.26	12.09 ± 0.705 ¥ *	7.07 ± 0.31
	D+30	6.89 ± 1.35	8.45 ± 0.75	9.16 ±1.25	6.79 ± 0.67

*: Significant difference in comparison to control group
¥: Significant difference in comparison to D0

This table shows that the administrations of AEPN to rats for seven (07) consecutive days at 100mg/Kg BW doesn't induce any change in kidney function biomarker (Urea, Creatinine). At 1000mg/Kg BW and 2500mg/Kg BW, we observed changes (in comparison to D0 but not versus control group) in those parameters that have been normalized after the observation time (D+30). In animals, urea is produced by the catabolim of protein and its elimination in urine is considered as removal of toxic ammonia from the body. Creatinine result from muscle activity with a relative constant rate in body. As urea, creatinine is filtered out of the blood by the kidneys.[16] An increase in Urea and Creatinine level in blood, due to their abnormal secretion by kidney, suggests renal dysfunction that could be attibuted to the loss of contact between podocytes pedicles and glomerular bassement membrane.[7] Morever increased urea levels are associated with nephritis, renal ischemia and urinary tract obstruction[16] but our study shows a reduction in ureamia and could suggest a nephroprotection role of AEPN. AEPN decreased ASAT level when administrated at 100mg/Kg BW and increased ALAT level at 1000mg/Kg BW. No significative change in serum of ALAT and ASAT under 2500mg/Kg BW was oberved after Seven days of extract adiministration and also at the end of experimentation. Interestingly, in all group after the 30 days of observation, all biochemical parameters normalized. we could conclude that AEPN doesn't have harmful on liver function. Howover, histological and deep biochemically investigation on those vital organs could contribute to safety use of P. nudum. The observation of rat behaviour after extract administration shows no physical troubles and no death during the experimentation. In perspective, we plan to investigate the toxicity of AEPN in mice, to assess its in vivo antioxydant property and its pharmacological effect against malaria.

CONCLUSION

Our study is the first reporting a toxicological study of P. nudum in rat. It reveals that AEPN contains different secondary metabolites and possess not less important in vitro anti-radical scavenging activity with DPPH and hydroxyle radical. The oral administration of the AEPN showed few changes in kidney and liver function biomarkers but didn't affection those organs function for long time upto 2500 mg/Kg of BW. Further studies are require before concluding to less toxicity of AEPN. For instant, the use of P. nudum for different purpose should be with much precaution

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