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PHARMACOGNOSTIC AND TOXICITY EVALUATION OF THE STEM BARK OF *TABERNAEMONTANA PACHYSIPHON* STAPF. (APOCYNACEAE).

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ABSTRACT
Various claims have been made on the ethno-medicinal uses of the stem bark of *Tabernaemontana pachysiphon*. It is therefore necessary to establish its pharmacognostic characteristics that will help in its identification and standardization. In this study, the pharmacognostic characteristics (phytochemistry, macroscopy, microscopy, chemo-microscopy, quantitative evaluation, proximate and preliminary Thin Layer Chromatography analyses) and toxicity potential of the stem bark were evaluated using standard methods of analysis.
Phytochemical analysis revealed the presence of carbohydrates, reducing sugars, alkaloids, flavonoids, saponins and cardiac glycosides. The stem bark has a bitter taste, flat in shape when fresh and curved when dry, greyish brown in colour on the outer surface and brown on the inner surface. It is longitudinally fissured and lenticels are present. Microscopic examination revealed the presence of cork cells, fibres, sclereids and calcium oxalate crystals. Chemo-microscopic tests showed the presence of lignin, calcium oxalate crystals, mucilage, cellulose and oil. Quantitative parameters (% $^{w}/_{w}$ ± S.E.M) were as follows: moisture content 4.68 ± 0.01; total ash value 8.98 ± 0.12; acid insoluble ash value 0.54 ± 0.01; water soluble ash value 2.05 ± 0.01; alcohol soluble extractive value 3.10 ± 0.01 and water soluble extractive value 0.42 ± 0.01. Proximate analysis of the powdered drug gave 16.59 % protein, 1.90 % fibre, 0.10 % fat, 67.75 % carbohydrate and 4.68 % moisture. Total gross energy was estimated to be 338.26kcal while preliminary thin layer chromatography (TLC) showed three spots. The aqueous extract of the stem bark, at a dose of 5 g/kg, caused neither death nor any observable symptoms of toxicity, after 24 hours.
These validated pharmacognostic standards will be useful towards proper identification of a closely related sample of the plant material and in the production of the Nigerian Pharmacopoeia of Nigeria medicinal plants.

Keywords: *Tabernaemontana pachysiphon*, Stem bark, Pharmacognostic standards, Acute toxicity test.

INTRODUCTION

Tabernaemontana pachysiphon Stapf. (Apocynaceae) is an African plant. It is commonly called "Giant pin wheel flower" (English) (Leeuwenberg and Kupicha, 1985); "ibu" (Edo); "oogele" (Igala); "pete-pete" (Igbo) and "abododo" (Yoruba) (Burkhill, 1985).

Tabernaemontana pachysiphon is a shrub or small tree that grows up to 15m in height with trunk up to 40 cm in diameter. Th[63]es are opposite, simple and entire. It has a petiole 6 - 20mm long, blade broadly to narrowly elliptical, 10 – 50cm X 5 – 26cm; base cuneate, apex acuminate to acute, pinnately veined with 7 - 16 pairs of lateral veins. Inflorescences mostly long-pedunculate and are few to many flowered. Flowers are bisexual, regular and sweet-scented. The fruit consists of two free almost globose follicles, 7

– 15cm in diameter, pale green and several to many seeded. It thrives in the under-storey of light forest, bush or riverine forest, from sea level up to 2200m altitude (Leeuwenberg and Kupicha, 1985; Elia, 2006).

The plant has many applications in ethnomedicine and is also economically important (Elia, 2006; Gill, 1992; Watt and Breyer-Brandwijk, 1962; Omino and Kokwaro, 1993).

It is no doubt that Africa (Nigeria in particular) is blessed with abundant plants whose medicinal potentials are not yet tapped. As a contribution to health care delivery, the scientist has the task of compiling and evaluating these commonly used medicinal plants and establishing their botanical, chemical and biological profiles, including where possible their uses and dosages towards their pharmacopoeia listing. It is in the light of these that studies of the stem bark of *T. pachysiphon*, a popular herbal medicine material, was undertaken with the objectives of establishing the pharmacognostic profile (phytochemistry, macroscopy, microscopy, chemomicroscopy, quantitative parameters, proximate and preliminary thin layer chromatography) of the genuine stem bark; determining the level of safety and to document the standard diagnostic features so obtained.

MATERIALS AND METHODS

Plant Material

The plant material was collected from the wild in November 2006, with the assistance of an herbalist from Iyowa village, in Ovia North East Local Government Area, Edo State Nigeria. Herbarium specimens were prepared. One was taken to the Nigeria Natural Medicine Development Agency (NNMDA), Lagos for initial identification; and another to Forest Research Institute of Nigeria (F.R.I.N), Ibadan for confirmation of identity by the Taxonomist, where voucher specimen number F.H.I 107750 was assigned. The stem bark was cut into small pieces, air dried for twelve (12) days, powdered with the aid of a mortar and pestle, and sieved with a filter of 8 inch wire mesh. The powder was stored in an air tight bottle till required for analysis.

Pharmacognostic Studies

Phytochemical screening

Tests were carried out on portions of the powdered plant material applying standard phytochemical screening methods (Sofowora, 1984; Evans, 2002). Bioactive agents tested for include glycosides, saponins, tannins, flavonoids and alkaloids.

Macroscopy

Macroscopical and organoleptic features of the bark noted were as follows; shape, colour (inner and outer surfaces), taste, fracture, presence or absence of lenticels, condition, presence or absence of fissures (Evans, 2002).

Microscopy

Microscopical features of the plant material were studied. The standard technique of clearing in chloralhydrate solution and mounting in glycerin was done. The sections were then observed under a microscope (African Pharmacopoeia, 1986). Presence or absence of features; cork cells, sclereids, fibres as well calcium oxalate crystals, were noted.

Chemomicroscopical examination

Examination of the powder for starch grains, lignin, mucilage, oil, calcium oxalate crystals, cellulose and hemicellulose were carried out (Evans, 2002).

Quantitative standards

Quantitative values were established. The parameters determined were; Moisture content, total ash, acid insoluble ash, water soluble ash, alcohol (99.8% ethanol) and water soluble extractive values (Africa Pharmacopoeia, 1986; British Pharmacopoeia, 1988).

Proximate analysis

These were determined using standard methods (AOAC, 1984; Pearson, 1976; Muller and Tobin, 1980; Osborne and Voogt, 1978). The nutritive constituents of the powdered plant material determined include the following: protein content, lipid content, fibre content, ash content, carbohydrate content and gross energy.

Preliminary Thin Layer Chromatography

This was done in an attempt to determine the possible number of alkaloids present in the plant extract. Five grams of the powdered material was treated with 40 % calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 20 ml portions of chloroform. The extracts were combined and concentrated to 5 ml. The chloroform extract was then spotted on thin layer plates.

A slurry of 1:2 ratio triturate of silica gel GF_{254} in distilled water was coated on glass plates (5 x 20 cm), using a spreader to give 0.50 mm thickness. The plates were allowed to air dry for 1 hour and activated in a thermostatically regulated oven at a temperature of 110^{o}C for 30 mins. Different tanks containing different solvent systems were prepared and used, from which the best was selected. In each case, 100 ml of the solvent mixture was used. The solvent systems were measured and poured into chromatographic tanks and the tank covered with a glass plate and then left for the solvent to saturate it. After development, the plates were brought out of the tank, allowed to dry and first observed in day light, then under Ultra-Violet (UV) light at 254 nm wavelength, then sprayed with Dragendorff's spray reagent. The distances from the origin of the various spots were noted and both the RF and HRF values were calculated from the formula respectively.

RF = Distance moved by spot / Distance moved by the solvent front.

HRF (%) = $\frac{\text{Distance moved by spot}}{\text{Distance moved by the solvent front}} \times 100$

Pharmacological evaluation

Plant extract

The powdered stem bark (200 g) was extracted using decoction method by boiling with water (750 ml) for 30 mins, allowing it to stand for 5 mins and then filtered. The filtrate was concentrated using an evaporating dish on a water bath, to yield a concentrate of 17.98 g. This residue was stored in an amber coloured bottled and preserved in the refrigerator until required.

Animals

Swiss albino mice (25-30 g) of both sexes were obtained from the animal house of the School of Medicine, Ambrose Alli University, Ekpoma; Edo State, Nigeria. The animals were kept in the animal house of the Department of Pharmacology, Faculty of Pharmacy, University of Benin. Prior to use, they were fed with Grower's mash chicken feeds, obtained from the Bendel Feed and Flour Mills Ewu, Edo State and had water *ad libitum*. The animals were allowed 14 days to acclimatize. Ethical standard with regards to the use of animals for experimental purpose was adhered to based on approval by the Ethical

Committee, Faculty of Pharmacy, University of Benin, Benin City.

Acute toxicity test

The aqueous extract was administered to mice through the oral route using the gastric tube. The animals were shared into six groups of five animals per group and treated with dose of 250, 500, 1000, 2000, or 5000 (mg/kg) of the extract or 10 ml/kg distilled water (vehicle). Animals were allowed feed and water *ad libitum*. Observations were made for 24 hrs for acute effects such as motor activity, tail erection, startle reaction, abdominal gripping, tremors, lacrimation, salivation, diarrhoea, excess curiosity, convulsions and any death. These observations were also made daily for a total of 14 days, for any delayed toxicity post drug administration (Dietrich, 1983).

RESULTS

At F.R.I.N, Ibadan; the plant material was confirmed as *Tabernaemontana pachysiphon* and voucher number F.H.I 107750 assigned by the Taxonomist, Mr. P.K Odewo. A herbarium specimen was deposited at F.R.I.N while another was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin.

The phytochemical analysis revealed the presence of carbohydrates, reducing sugars, saponin glycosides, cardiac glycosides, flavonoids and alkaloids. Anthracene glycosides, cyanogenetic glycosides and tannins were not present in the powdered plant material (Table 1).

Macroscopically, the bark drug is bitter to the taste, curved in shape when dry, grayish brown in colour on the outer surface and brown on the inner surface. It is longitudinally fissured and lenticels are present.

Microscopic examination revealed the presence of cork cells, fibers, sclereids and prismatic calcium oxalate crystals (Figure 1).

Chemomicroscopic tests showed the presence of lignin, calcium oxalate crystals, mucilage, cellulose and oil. Starch and hemicellulose were not present in the powdered plant material (Table 2).

In the powdered plant sample, moisture content, total ash value, acid insoluble ash value, water soluble ash value, alcohol soluble extractive value and water soluble extractive value were found to be 4.68% $^{w}/_{w}$; 8.98% $^{w}/_{w}$; 0.54% $^{w}/_{w}$; 2.05% $^{w}/_{w}$; 3.10% $^{w}/_{w}$ and 0.42% $^{w}/_{w}$ respectively (Table 3).

Proximate analysis of the crude drug for its nutritive constituents gave 16.59% protein; 1.90% fiber; 0.10% fat; 67.75% carbohydrate; 4.68% moisture and 8.98% ash (Table 4).

In preliminary thin layer chromatography, the tank containing Ethylacetate: Ethanol: Ammonia as solvent system in ratio 2: 3: 1 gave the best result, showing three spots. In daylight, the spots were colourless and under UV light at 254 nm wavelength, they had colours. When the TLC plates were sprayed with Dragendorff's reagent, they appeared as reddish brown spots on a yellow background, which confirmed the presence of alkaloids in the plant material (Table 5).

In the test for acute toxicity, increasing doses of the aqueous extract up to 5000 mg/kg produced no death and there were no observable signs of toxicity manifested on administration of the extract after 24 hours.

DISCUSSION

Phytochemical screening was carried out to establish the presence of bioactive agents in the powdered plant material (crude drug). In the phytochemical screening of the plant material, the production of a violet ring

TABLE 1: Summary of results of phytochemical tests.

CONSTITUENTS	STATUS
Carbohydrates	Present
Reducing sugars	Present
Saponin glycosides	Present
Anthracene derivatives	Absent
Cyanogenetic glycosides	Absent
Cardiac glycosides	Present
Tannins	Absent
Flavonoids	Present
Alkaloids	Present

Figure 1A: Prismatic calcium oxalate crystals.

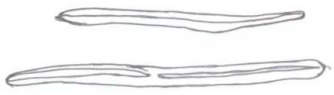

Figure 1B: Pericyclic fibres.

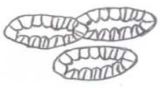

Figure 1C: Sclereids.

Figure 1D: Cork cells.

Figure 1: Microscopical features of the powdered stem bark of *Tabernaemontana pachysiphon*. (MAG. X 400).

at the interface of the aqueous extract of the plant material and the concentrated sulphuric acid layer indicated the presence of carbohydrates. A brick red precipitate was formed when the extract was added to Fehling's solutions (A and B) and heat applied, thus indicating reducing sugars in glycosides. However, of all the tests performed for the specific glycosides, anthraquinone, saponin, cardiac and cyanogenetic; only saponin and cardiac glycosides gave the positive thick persistent froth result and positive Keller-Kiliani; Kedde and Salkowski's tests respectively. The presence of flavonoids was indicated by yellow colour obtained when the drug was tested with sodium hydroxide solution but turned colourless on treating with 1% aqueous hydrochloric acid (Evans, 2002). The absence of a blue–black or greenish precipitate, formed when water extract of the plant material is reacted with 5% ferric chloride test solution indicated the absence of tannins in the plant material. The formation of characteristic coloured precipitates when few drops of alkaloidal reagents (Mayer's; Hager's; Dragendorff's and Wagener's reagents) are added to the extract of the plant material indicated the presence of alkaloids.

Macroscopical characters of any medicinal plant are the first guide steps to identifying the plant, as these only ensure that the right morphological part has been collected.

The microscopical composition of a medicinal plant is of value in enhancing the identity of a plant. Macroscopical features are lost when the material is reduced into powder, therefore for proper identification; microscopical features of the powdered drug were also studied.

The cell wall may undergo changes that result in the deposition or incrustation of certain chemicals such

TABLE 2: Chemomicroscopical constituents of the powdered bark.

S/No.	TESTS	OBSERVATIONS	INFERENCES
i	Lignin	Red colour observed.	Lignin present.
ii	Starch	No blue black colour observed.	Starch absent.
iii	Calcium oxalate crystals	Prisms of calcium oxalate crystals observed.	Calcium oxalate crystals present
iv			
v	Mucilage		
vi	Cellulose Hemicellulose	Pink colour observed.	Mucilage present.
		Blue colour observed.	Cellulose present.
vii	Oil	No red colour observed.	Hemicellulose absent.
		Small patches of pink colour observed.	Fixed Oil present

TABLE 3: Summary of results for quantitative determinations.

PARAMETERS	VALUES (% $^{w}/_{w}$)
Moisture content	4.68 ± 0.01
Total ash	8.98 ± 0.12
Acid insoluble ash	0.54 ± 0.01
Water soluble ash	2.05 ± 0.01
Alcohol soluble extractive	3.10 ± 0.01
Water soluble extractive	0.42 ± 0.01

Values are Mean ± S.E.M. n = 10.

TABLE 4: Results of proximate analysis.

PARAMETERS	VALUES (%)
Protein	16.59 ± 0.07
Fat	0.10 ± 0.02
Fibre	1.90 ± 0.04
Ash	8.98 ± 0.12
Carbohydrate	67.75 ± 1.15

Values are Mean ± S.E.M. n = 3.

GROSS ENERGY ESTIMATION = 338.26 kcal.

Result of preliminary thin layer chromatography (TLC).
Adsorbent: Silica gel GF_{254}
Thickness of adsorbent: 0.50 mm
Solvent system: Ethylacetate: Ethanol: Ammonia
Solvent ratio 2: 3: 1
Distance moved by developing solvent: 10 cm
Development time: 48 mins

Table 5: RF values, HRF values and colour reactions of the extract of *T. pachysiphon* stem bark using solvent system Ethylacetate: Ethanol: Ammonia (2: 3: 1).

Spots	Colour in day light	Colour in UV (254 nm)	Colour after Spraying with Dragendorff's reagent	RF Values	HRF Values
1	Colourless	Light green fluorescence	Reddish brown	0.10	10
2	Colourless	Light green fluorescence	Reddish brown	0.69	69
3	Colourless	Blue fluorescence	Reddish brown	0.97	97

as cellulose, lignin or hemicellulose in it. The detection of the presence of these chemicals (not observed by macroscopy and microscopy) is made possible by certain colour reactions with different reagents (chemomicroscopy). For example, the presence of lignin is detectable by the pink or red colour that is produced when phloroglucinol and hydrochloric acid is added to a cleared section or powder of the plant material.

Moisture content determination is of great significance, as the presence of excessive water in vegetable drugs, greater than the set limit will promote the growth of microbes, fungi or insects and the hydrolysis of constituents leading to a deterioration of the drug. Total ash value which comprises both the natural or physiological ash and the non-physiological ash values may vary from specimens of genuine drugs due to the variable natural or physiological ash (derived from the plant tissue itself or that from mineral elements) and non-physiological ash (derived from extraneous matter especially sand). In such cases, the ash obtained is treated with acid in which most of the natural ash is soluble leaving the silica as acid-insoluble ash which represents most of the ash from the contaminating soil (Wallis, 1985). The water soluble ash is used to detect the presence of exhausted materials substituted for the genuine drugs. Such substitutions result in the lowering of this value. The ethanol and water soluble extractives values are used in the determination of the amount of constituents which are extractable by the solvent under specified conditions. It also helps in confirming the genuity of the plant material by guarding against supply of exhausted drug.

The stem bark of *T. pachysiphon* contained some amount of basic food nutrients such as proteins, fats, carbohydrate and fibre (Table 6). Dietary fibre enhances frequent waste elimination and promotes bowel regularity. Fibre has a physiological effect on the gastrointestinal function of promoting the reduction of tracolonic pressure. It also has a biochemical effect on the absorption and reabsorption of bile acids and hence, lowers cholesterol pool (Okwu, 2006). Food fibre also aids absorption of trace elements in the gut (Kelsay, 1981). Proteins contain amino acids utilized by the cells of the body to synthesize all the numerous proteins required for the function of the cell and also to furnish energy (Robinson, 1978). Total gross energy was calculated to be 338.26 kcal. This energy value is due to the high carbohydrate content which is present mainly as cellulose and glycosides. In plant materials, the nitrogen free extract (carbohydrate content) could be as much as 68.6% as in the leaves, bark and pods of various *Acacia species* (Dougal *et al.*,1964; Gohl, 1981).

The thin layer chromatography not only confirmed the presence of alkaloids in the plant material but also, showed that the plant material could be a source of a number of useful medicinal alkaloids.

Acute toxicity studies of a drug or extract are carried out in order to evaluate its safety for the purpose of medication. Acute toxicity indicates the toxic effects produced by a single dose or multiple dose of a compound or extract given during a 24 hr period. In this study, no observable signs of toxicity manifested on administration of the extract after 24 hours. The LD_{50} could not be calculated since no death was observed. This therefore implies that, the aqueous extract is relatively safe, as its LD_{50} is greater than 5 g/kg.

CONCLUSION

The standardization of the stem bark of *T. pachysiphon* is an integral part of establishing the correct identity of the crude drug. Before any drug can be included in the pharmacopoeia (for example, Nigerian Pharmacopoeia), these pharmacognostic parameters and standards must be established. Thus, these validated pharmacognostic standards will be useful towards proper identification of a closely related sample of the plant material and in the production of the Nigerian Pharmacopoeia of Nigeria medicinal plants. Also, the aqueous extract of the plant material is relatively safe. However, it is necessary to subject the plant material to more chromatographic analyses (so as to obtain a good representative of the number of compounds present in the plant part) and to longer periods of toxicity studies (subacute and chronic), using different animal species if possible. This study thus forms a primary platform for further pharmacological studies.

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