

Full Length Research Paper

Pharmacognostic evaluation and antisickling activity of the leaves of *Peperomia pellucida* (L.) HBK (Piperaceae)

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Traditional healers have been using condiments from natural sources as antisickling remedies in Southern Nigeria. This study was aimed at establishing the pharmacognostic profile and investigating the antisickling activity of the leaves of *Peperomia pellucida* (L.) HBK (Piperaceae). Evaluation of the fresh, powdered and anatomical sections of the leaves were carried out to determine the macromorphological, micromorphological, chemomicroscopic, numerical and phytochemical profiles. Chemical tests were employed in phytochemical investigations. Evaluation of the antisickling activity involved the inhibition of sodium metabisulphite-induced sickling of the HbSS red blood cells obtained from confirmed sickle cell patients who were not in crises. Macro and microscopical studies gave results that could serve as a basis for proper identification, collection and investigation of the leaves of *P. pellucida*. Phytochemical evaluation revealed the presence of alkaloids, tannins, flavonoids, saponins and cardiac glycosides. Percentage sickling inhibitions of the aqueous methanol extract of *P. pellucida* were significant all through the period of assay $p < 0.05$ compared to normal saline. The maximum percentage inhibition of sickling exhibited by the 500 mg/ml of the extract was 57.5% at 90 min incubation. These results showed that *P. pellucida* has a role in the treatment of sickle cell disorders and a candidate for further investigations.

Key words: *Peperomia pellucida*, Piperaceae, pharmacognostic evaluation, sickle cell disorders.

INTRODUCTION

The use of and search for drugs and dietary supplements derived from plants have increased in recent years. Pharmacologists, microbiologists, botanists and natural product chemists are combing the earth for phytochemicals and leads that could be developed for treatment of diseases. Many of the Pharmaceuticals currently available to Western Physicians have a long

history of use of herbal remedies. Despite all the progress in synthetic chemistry and biotechnology, plants are still an indispensable source of medicinal preparations, both for prevention and cure. The World Health Organization (WHO) estimates that 80% of the world's population presently uses herbal medicines for some aspects of primary healthcare (Srivastara et al., 1996).

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The occurrence of sickle cell disease (SCD) is predominantly in persons of African origin, but it also affects persons of Mediterranean, Caribbean, South and Central American, Arab and East Indian origins. In Nigeria, the most common type of SCD is the homozygous (HbSS) form that is, sickle cell anaemia (SCA). Estimates show that 25% of the Nigerian population are "carriers" of the sickle cell trait that is, HbAS (Coker et al., 2006).

Despite a variety of antisickling agents acting at different levels of the sickling mechanism, there is still a paucity of antisickling medicines. This is because of the potential toxicities associated with most of these agents. Apart from the general mutagenic and carcinogenic tendencies of gene modifiers, hydroxyurea causes bone marrow suppression which greatly limits its use (Strouse et al., 2008). It is indeed a sad note that almost a century of search both for curative means and disease modifying isolated xenobiotics has been almost futile. On the other hand, this is gradually paving way for the consideration of condiments from natural sources as antisickling remedies. The increasing interest in these condiments is not unconnected with the general innocuous nature of their sources, which most often are herbs and even at times food crops. For example, some plants that already have acclaimed antisickling properties are: *Cissus populnea*, *Xanthoxylum xanthoxyloides*, *Terminalia cartappa* and *Cajanus cajan* (Adesanya and Sofowora, 2006). Most of these medicines come in the form of herbal formulations comprising two or more of these plant materials. *Peperomia pellucida* (L.) HBK (Piperaceae) is one of the recipes being used by traditional healers in Southern Nigeria in managing sickle cell anaemia, though without scientific basis.

The genus *Peperomia* (Family: Piperaceae) comprises more than 1000 species, including *P. pellucida*, which is found in various shaded, damp habitats all over Asia and America. It grows in clumps, thriving in loose, humid soils. It is an annual or short-lived perennial, erect or decumbent, freely branched glabrous plant (Moreira et al., 1997). Common names include pepper elder, silver brush (North America), suna kosho (Japan), coracozinho or little heart (Brazil), potpopot (Oceania), cang cua (Vietnam) and pak krasang (Thailand) (Arrigoni-Blank et al., 2002).

A study has identified 71 compounds isolated from the essential oils of 10 Piperacea species. Sesquiterpenes appear to be the major chemical constituent in the essential oils. Carotol (13.4%) was the major hydroxylated sesquiterpene in chemical analysis of *P. pellucida*. Flavonoids, phytosterols, arylpropanoids, substituted styrenes and a dimeric ArC₂ compound (Pellucida A) have been isolated (Bayma et al., 2000; Xu et al., 2006). The analgesic and anti-inflammatory activities of *P. pellucida* have been reported (Dmitrieva et

al., 2000). The analgesic effect is associated with prostaglandin synthesis interference. Chloroform extract from the dried leaves have antifungal activity against *Trichophyton mantagrophytes* (Ragasa et al., 1998) and crude methanol extract has broad spectrum antimicrobial activity (Khan and Omoloso, 2002).

This study is the pharmacognostic and biological evaluation of *P. pellucida* (L.) HBK (Piperaceae), one of the recipes which has been used with acclaimed success by traditional healers in Nigeria in managing sickle cell anaemia. The antisickling investigations will provide supportive scientific evidence in favour or otherwise of its continuous usage. Also, the standardization of a crude drug is an integral part of establishing its correct identity. Before any crude drug can be included in an herbal pharmacopoeia, some Pharmacognostic parameters and standards must be established.

MATERIALS AND METHODS

Preparation of plant extract

The leaves of *P. pellucida* (L.) HBK (Piperaceae) were collected in Ugbowo area of Benin City, Edo State, Nigeria. The plants were authenticated by the curator at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City where voucher specimens were deposited. The fresh leaves were air-dried for 72 h and powdered using an electric mill.

Macroscopy

The following macroscopic characters for the fresh leaves were noted: size and shape, colour, surfaces, venation, presence or absence of petiole, the apex, margin, base, lamina, texture, odour and taste (Wallis, 1985; Evans, 2006).

Microscopy

The outer epidermal membranous layer (in fragments) were cleared in chloral hydrate, mounted with glycerin and observed under a compound microscope. The presence/absence of the following was observed: epidermal cells, stomata (type and distribution) and epidermal hairs (types of trichomes and distribution). The transverse sections of the fresh leaves through the lamina and the midrib as well as a small quantity of the powdered leaves were also cleared, mounted and observed (African Pharmacopoeia, 1986).

Chemomicroscopic examination

Examination of the powder for starch grains, lignin, mucilage, calcium oxalate crystals, cutin and suberin were carried out using standard techniques (Evans, 2006).

Phytochemical investigation

Chemical tests were employed in the preliminary phytochemical screening for various secondary metabolites such as tannins

(phenazone; iron complex; formaldehyde and modified iron complex tests were carried out on the aqueous extract to detect the presence of hydrolysable, condensed and pseudo tannins), cardiac glycosides (Keller - Killiani and Kedde tests were carried out on the methanolic extract to detect the presence of a deoxy sugar, whose natural occurrence is to date, known only in association with cardiac glycosides and to indicate the presence of a lactone ring on the cardenolides respectively), alkaloids (Mayer's, Dragendorff's, Wagner's and 1% picric acid reagents to detect the presence of alkaloidal salts and bases), saponins glycosides (frothing of the aqueous extract when shaken and haemolysis test on blood agar plates were carried out to indicate and confirm the presence of saponins), anthracene derivatives (Borntrager's test for combined and free anthraquinones, where aglycones were extracted using chloroform and shaken with dilute ammonia) and cyanogenetic glycosides (sodium picrate paper test were used to test for the presence of hydrocyanic acid in the sample. Conversion to sodium isopurpurate indicates the presence of cyanogenetic glycosides) (Evans, 2006; Brain and Turner, 1975; Ciulei, 1981; Harborne, 1992).

Quantitative investigation

Quantitative investigations to determine moisture content, total ash, acid-insoluble ash, water-soluble ash, alcohol (90% ethanol) and water soluble extractive values were carried out using standard procedures (African Pharmacopoeia, 1986; British Pharmacopoeia, 1988).

Moisture content

The powdered drug (2.0 g) was weighed into a clean crucible of known weight. After oven drying at 105°C for 5 h and cooled, the crucible was weighed again to determine weight loss in the powdered drug. The average percentage weight loss, with reference to the air dried powdered drug was determined for thirty replicates.

Total ash determination

The crucibles were washed thoroughly, dried in hot oven at 100°C, cooled in desiccators and weighed. A 2.0 g portion of each of the samples were weighed into the crucible and put in the furnace. Heating was started gradually until temperature of 600°C was reached. This temperature was maintained for 6 h. The crucible was then put inside the desiccators and cooled. After cooling the sample was reweighed and the percentage ash calculated.

$$\% \text{ Ash} = \frac{W-Z}{N} \times 100$$

W = weight of the crucible and ash; Z = weight of empty crucible; N = Weight of sample.

Acid-insoluble ash determination

The total ash was treated with 25 ml dilute hydrochloric acid in a crucible, boiled gently for 5 min while covered with a watch glass and filtered through ashless filter paper (Whatman No.1) of known weight. The crucible was washed with hot water and the washings

passed through the filter paper. This was continued until the filtrate became neutral to litmus paper. The paper with the insoluble matter was dried to a constant weight at 105°C. The weight of the insoluble matter was determined by subtracting the weight of the filter paper from the dry weight of the filter paper containing the insoluble ash. The percentage of the acid-insoluble ash with reference to the air-dried material was calculated.

Water-soluble ash determination

The water-soluble ash was determined by adding 25 ml of water to the ash. After boiling gently for 5 min, the content of the crucible was filtered through previously weighed dried ashless filter paper. After washing the residue with hot water, the filter paper was dried in an oven at 105°C until a constant weight was obtained. The weight of the residue was obtained by subtracting the weight of the dry filter paper from the weight of the residue and the filter paper. The weight of the water soluble ash was then obtained by subtracting the weight of the insoluble ash (that is, the residue) from the weight of the total ash. The percentage of water soluble ash with reference to the air-dried powdered material was then determined.

Alcohol soluble extractive value

Powdered leaf drug (5.0 g) was weighed into a 250 ml stopper conical flask. Ethanol 90% (100 ml) was added to the conical flask and stoppered. The flask was shaken in a mechanical shaker for 6 hours and then allowed to stand for 18 h. The extract was filtered by suction filtration using a Buckner funnel. The weight of a heated cooled flat bottom porcelain crucible was accurately determined. The filtrate was poured into weighed crucible and evaporated to dryness at 100°C. The residue was dried to constant weight and the final weight noted. The weight of the residue obtained from the extract was determined by subtracting the constant weight of crucible from the residue. The alcohol extractive was then calculated with reference to the initial weight of the powdered drug and expressed as percentage.

Water soluble extractive value

The aforementioned experiment was repeated using chloroform-water.

Antisickling screening

HbSS blood samples

HbSS blood samples were collected by venipuncture from confirmed sickle cell patients not in crises on their clinic days at the Consultant Outpatient Department (COPD) of the University of Benin Teaching Hospital, Benin City, Nigeria. None of the patients used was recently transfused with HbAA blood.

Antisickling activity evaluation

The evaluation of the leaf extract of *P. pellucida* for antisickling activities was carried out using a modified method of Moody et al. (2003). Venipuncture blood samples from sickle cell anaemia patients not in crises were collected into EDTA bottles. Collected

Table 1. Numerical data of leaves of *Peperomia pellucida*

Parameter	Mean \pm SEM (% w/w)
Moisture content*	10.90 \pm 0.39
Total ash*	14.25 \pm 0.24
Acid – insoluble ash [†]	1.25 \pm 0.11
Water – soluble ash [†]	9.75 \pm 0.19
Alcohol – soluble extractive*	2.53 \pm 0.02
Water – soluble extractive*	4.08 \pm 0.05

*n = 30, [†]n = 15.**Table 2.** Phytochemical constituents of *P. pellucida* leaves.

Classes of secondary metabolites	inferences
Alkaloids	+
Tannins	+
Flavonoids	+
Anthracene derivatives	-
Saponin glycosides	+
Cardiac glycosides	+
Cyanogenetic glycosides	-

- = absent; + = present

samples were centrifuged to remove the serum. The resulting packed erythrocytes were washed three times with sterile normal saline and centrifuged each time to remove the supernatant. 0.5 ml of the washed erythrocytes was mixed each with 0.5 ml of the different concentrations of the aqueous methanol extract (100 to 500 mg/ml) in uncovered test tubes. Samples were taken from the different mixtures and the remaining portions of the mixtures incubated for 3 h, shaking occasionally. 0.5 ml of 2% sodium metabisulphite was added to each mixture to deoxygenate the system, mixed thoroughly and sealed with liquid paraffin. Samples were taken in quadruplicates from the different mixtures at 0 minutes and at subsequent 30 min interval until seven readings were obtained. Each sample was smeared on a microscopic slide, fixed with 95 % methanol, dried and stained with giemsa stain. Each slide was examined under the oil immersion light microscope and counting of 100 red cells in each sample. The numbers of both sickled and unsickled red blood cells were counted and the percentage of unsickled cells determined.

Statistical analysis

Data are expressed as mean \pm standard error of mean (SEM). The differences between the means were analyzed using one way analysis of variance (ANOVA). Values of $P < 0.05$ were taken to imply statistical significance between compared data.

RESULTS

Macroscopic description of the leaves of *P. pellucida*

The leaf was dark green in the upper surface and light

green in the lower surface. It was turgid when fresh but soft few hours after collection. It was a simple leaf, with a cordate shape. The margin was entire, apex was acuminate, base truncate and venation was reticulate. Average leaf size was 3.3 cm \pm 0.7 (length) and 3.0 cm \pm 0.4 (breadth). The fresh leaf had a carrot-like taste and the odour was characteristic.

Microscopic Description of the leaves of *P. pellucida*

Micromorphological features revealed that anticlinal walls were thick and wavy. Stomata were present in both lower and upper epidermi. The stoma was surrounded by three or four epidermal cells with one of the subsidiary cells markedly smaller than the others (anisocytic arrangement). There were numerous uni- and multi-seriate covering trichomes present on both surfaces. A transverse section of the leaf across the mid-rib showed an upper and lower epidermi consisting of cells of similar sizes. It had a bifacial surface that is, there were two different surfaces with different identities, hence dorsiventral. The mesophyll consisted of a palisade and the spongy mesophyll, embedding a crystal sheath. There were crystal clusters of calcium oxalate in the spongy mesophyll. The mid-rib bundle was surrounded by a zone of collenchyma on both surfaces. The phloem vessels embedded the xylem vessels. Chemo-microscopic examination of the leaves revealed the presence of starch, mucilage, calcium oxalate crystals and cellulose.

Numerical data of the leaves of *P. pellucida*

The moisture content of *P. pellucida* which fell within the Pharmacopoeia limit, the ash values as well as the amount of constituents which were extractable by methanol and water under specified conditions are presented in Table 1.

Phytochemical screening

Phytochemical screening of the leaves of *P. pellucida* for secondary plant metabolites revealed the presence of alkaloids, tannins, flavonoids, saponins and cardiac glycosides (Table 2).

Sickling inhibitory activities of crude extracts of *P. pellucida*

Percentage sickling inhibition of the various doses of *P. pellucida* was significant all through the period of assay p

Table 3. The sickling inhibitory activities of *Peperomia pellucida* leaves at different concentrations.

Time of incubation (min)	Percentage inhibition (%)						
	Normal saline	PHBA	100 mg/ml	200 mg/ml	300 mg/ml	400 mg/ml	500 mg/ml
0 time before incubation	30.2±2.26	40.3±1.94	45.1±1.43	48.4±2.30	47.9±1.33	52.3±0.94	50.9±1.24
0 time after incubation	26.1±1.73	52.6±0.91	48.4±2.12	47.1±2.17	45.7±1.72	48.6±1.21	45.5±0.86
30	26.2±2.16	61.3±1.68	45.2±1.65	48.5±1.41	48.6±1.41	51.1±1.17	49.7±1.37
60	26.9±1.44	64.6±1.85	49.8±2.07	51.6±1.89	52.7±2.35	53.6±1.88	51.7±1.12
90	22.5±1.19	59.7±1.83	51.7±1.81	49.4±1.83	56.2±1.48	55.2±1.49	57.2±2.05
120	28.4±2.74	46.5±2.04	47.6±0.93	49.0±1.34	49.4±1.93	50.6±0.82	53.4±0.94
150	26.7±1.29	40.8±2.33	40.2±2.14	46.6±2.08	48.4±0.84	46.4±1.44	48.0±1.19
180	24.3±0.65	38.3±2.48	38.4±1.68	37.9±1.53	39.3±1.07	41.7±1.11	44.4±1.53

< 0. 05 compared to normal saline n = 4 (Table 3).

DISCUSSION

P. pellucida (L.) HBK is currently being used in the treatment of sickle cell disorders and various disease conditions without standardization. Before any crude drug can be included in a herbal pharmacopoeia, pharmacognostic parameters and standards must be established (Chandel et al., 2011). The results of these pharmacognostic investigations could therefore serve as a basis for proper identification, collection and investigation of the plant. The macro- and micro-morphological features of the leaf described distinguishes it from other members of the genera. Chemomicroscopy and numerical data are parameters that are unique to the plant and are required for correct identification. The numerical data were determined to assist in establishing the identity of crude drugs. Not only is the purchase of drugs which contain excess water uneconomical, but also in conjunction with suitable temperature, moisture will lead to the activation of enzymes and given suitable conditions, to the proliferation of living organisms (Evans, 2006). As most vegetable drugs contain all the essential food requirements for moulds, insects and mites, deterioration can be very rapid once infestation has taken place. The moisture content of *P. pellucida* obtained in the determination met the pharmacopoeia limits of water content for vegetable drugs, which is between 8 to 14% (African Pharmacopoeia, 1986). From the foregoing, the plant material can be conveniently stored at room temperature without the deterioration of its active constituents. The Pharmacological activities of a given plant are associated with the type and nature of secondary plant metabolites present. The need for phytochemical screening has become imperative, since

many plants accumulate biologically active chemicals in their tissues. Phytochemical evaluation of *P. pellucida* revealed the presence of alkaloids, tannins, flavonoids, saponins and cardiac glycosides.

The *in vitro* technique adopted in the antisickling efficacy bioassay was based in the simulation of the major *in vivo* sickling-precipitating factor (that is reduction of oxygen tension), using sodium metabisulphite as a physiologically acceptable reducing agent. The use of erythrocyte suspension instead of whole blood was particularly essential in ruling out the possibility of interactions of plasma component and products of their several immunological reactions and certain metabolic co-factors in general with the red blood cells (Coker et al., 2006). Such interactions could significantly affect the shape and size of red blood cells and in the process inadvertently produce false negative or false positive results. The aqueous methanol extracts of *P. pellucida* showed significant inhibitory effect at the concentrations (100-500 mg/ml) on sodium metabisulphite-induced sickling. Percentage inhibition increased with time of incubation up to 90 min.

Conclusion

The pharmacognostic parameters of *P. pellucida* which are being reported for the first time could be useful in its standardization. On the basis of the biological results, aqueous methanol extracts of *P. pellucida* have been found to possess an antisickling activity, which makes it a good candidate for further works.

Conflict of Interest

The authors have not declared any conflict of interest.

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Conflict of interest

Authors have none to declare.

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