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PHYTOCHEMICAL, ANTIBACTERIAL AND CYTOTOXIC EVALUATION OF RAPHIOSTYLIS BENINENSIS [HOOK F. EX PLANCH] STEM BARK EXTRACTS

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ABSTRACT

Methanol and ethyl acetate (stem-bark) extracts of *Raphiostylis beninensis* were investigated for their phytochemical, antibacterial and cytotoxic properties. Phytochemical analysis of ethyl acetate extracts of *R. beninensis* revealed alkaloids (1.10 ± 0.20 mg/kg), flavonoids (6.10 ± 0.10 mg/kg), saponins (5.30 ±0.15 mg/kg) and tannins (2.10 ± 0.20 mg/kg). Methanol of extract *R. beninensis* contained alkaloids (1.50 ±0.10 mg/kg), flavonoids (6.24 ± 0.20 mg/kg), saponins (7.50 ±0.10 mg/kg) and tannins (1.32 ± 0.03 mg/kg). The stem bark methanol and ethyl acetate extracts of *Raphiostylis beninensis* inhibited all tested strains *Escherichia coli, Bacillus subtilis, Salmonella typhii and Staphyllococcus aureus* at 20 mg/ml and 50 mg/ml respectively. The brine shrimp lethality test on the methanol and ethyl acetate extracts of *Raphiostylis beninensis* revealed cytotoxic activities with LC_{50} of 3.20 and 2.50 µg/ml.

KEYWORDS

Raphiostylis beninensis, Antibacterial Activity, Cytotoxic Property, Phytochemical, Methanol Extract, Ethyl Acetate Extract.

INTRODUCTION

Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. It has been noted that infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, in spite of tremendous growth in human medicine. Their impact is large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance[1]. Over the years, the medicinal and pharmaceutical potentials of plants have been studied and attributed to the various phytochemicals (secondary metabolites) accumulated in the various morphological parts of plants. This has led to the isolation of a great number of organic compounds [2]. The estimate of the World Health Organization (WHO) revealed presently that over 60% of the people in developing countries rely chiefly on traditional medicine for their primary health needs, of which a major portion involves the use of plant extracts or their active principles[3].

Raphiostylis beninensis Hook F. ex Planch (family – Icacinaceae), is one of the lesser-known species of the genus *Raphiostylis* that grows naturally as shrub in semi-deciduous, savannah forest area[4]. The family, to which *R. beninensis* belongs, consists of about 52 genera and over 400 species[3]. *R. beninensis* is a glabrous evergreen shrub, with smooth, dark-grey stem bark. Its leaves are purple-black when dried. The flowers are rather numerous in auxiliary fascicles, it flowers between December and January[5,6]. The seeds of *R. beninensis* are said to be edible. *R. beninensis* has many Nigerian vernacular names, depending on the location and usage. The plant is called *atapata* (Yoruba), *osumadin* (Benin), *kpokoto* (Ibos), *umeni* (Urhobos) and *kumeni* (Itsekiris).

A potential mosquito-repellant property has been demonstrated by the aqueous extracts of *R. beninensis* by the natives of Cote d'Ivouire[7]. The root, stem and leaves are boiled and drunk to kill and expel round worm[7]. In South-Western Nigeria, *R. beninensis* is reportedly used as a tonic for children between the ages of two to three years, and for treating a disease called *afun*- a diseased condition where entire skin turns white[8]. A root decoction of *R. beninensis* is used as a mouth wash, lotion for sores and in curing convulsion[9]. The family to which *R. beninensis* belong (Icacinaceae) has been reported to contain numerous bioactive phytochemicals [Nkafaniya *et* al., 2007; Gandiza *et al*., 1993].

In spite of the pharmacological and antimicrobial potentials of *R. beninensis,* no single compound has so far been isolated from it, except a report on the chemical characterization and antimicrobial potentials of oils obtained from the roots of *R. beninensis*[10]. In response to the folkloric utility of *R. beninensis* and in continuation of our search for bioactive phytochemicals in natural products, we investigated the stem bark extracts of *Raphiostylis beninensis* for phytochemical, antibacterial and cytotoxic properties.

MATERIALS AND METHODS

Collection of Plant Material

The roots, stems and leaves of *Raphiostylis beninensis* were collected from Olookemeji Forest Reserve, Eruwa in Ibarapa Local Government Area, Oyo State, Nigeria in June, 2008. The plant was identified by Mr. T. K. Odewo, a forester in the Herbarium section, Forest Research Institute of Nigeria (FRIN), Jericho, Ibadan, Oyo State, Nigeria. A voucher specimen with the collection number FHI 1120 was preserved in the herbarium section, FRIN.

Extraction of Plant Material

Stem barks of *R. beninensis* was air-dried on laboratory bench for two weeks and then ground into a uniform powder, using a Thomas Wiley machine, USA. The stem bark (1.2 kg) of *R. beninensis* was successively and exhaustively extracted with distilled n-hexane (bp. 66-68 ^{o}C) in an aspirator bottle fitted with extraction accessories. The residue was later re-extracted with distilled methanol (bp 64.5 ^{o}C). The stem methanol extract was concentrated under vacuum, at reduced pressure to give thick dark viscous solid (330 g). Water was added to 200 g of the stem methanol extract of *R. beninensis*, shaken thoroughly and later repartitioned with ethyl acetate (bp 77 ^{o}C), using a separating funnel to yield aqueous and ethyl acetate extracts of *R. beninensis*. The ethyl acetate extract was concentrated under vacuum to yield golden-yellow extract (20 g).

Phytochemical analysis of the extracts

Alkaloids

5 g of the samples were weighed into 250 ml beaker and 20 ml of 20% acetic acid was added and covered to stand for 4 hrs. This was filtered and the extract was concentrated using a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract and the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed[11,12].

Flavonoids

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whitman filter paper (25 mm, No 42). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed to a constant[13,14].

Tannins

5g of the sample was weighed into 100 ml plastic bottles. 50 ml of distilled water was added and shaken for 1hr. in a mechanical shaker. The mixture was filtered into 500 ml volumetric flask and made up to the mark. Then, 5 ml of the filtrate was pipetted out into a tube and mixed with 3 ml of 0.1M $FeCl_2$ in 0.1 M HCl and 0.08 M $K_2(FeCN)_6$. The absorbance was measured in a spectrophotometer at 605 nm wavelength within 10 minutes. A blank sample was prepared and the colour also developed and read at the same wavelength. A standard was prepared using tannin to get 100 ppm and measured[14].

Saponnins

Analysis of saponnins was carried out using the procedure used for tannins[14].

Biological Assays

Sources of Bacterial Strains and Antibacterial Assay

Clinical isolates of *Escherichia coli, Bacillus subtilis, Salmonella typhii and Staphyllococcus aureus* used for bioassay were obtained from the department of Microbiology, University of Ibadan, Ibadan Nigeria. The cork and bore diffusion method was used to determine the *in vitro* antibacterial activity of the methanol and ethyl acetate extracts of *R. beninensis*[15]. Pure culture of the bacteria organisms were

inoculated onto Mueller-Hinton Agar (Merck) and incubated for 24 hrs at 37 ^{o}C. About 5 discrete colonies were aseptically transferred using sterile wire loop into tubes containing sterile normal saline (0.85% NaCl) and were adjusted to a turbidity of 0.5 MarcFarland standards. The suspensions were then inoculated on the surface of sterile Mueller-Hinton Agar plates using sterile cotton swabs. A sterile 6 mm diameter cork borer was used to make holes (wells) into the set of inoculated Mueller-Hinton Agar. The wells were filled with different concentrations of the extract. The plates were incubated for 24 hrs at 37 ^{o}C, and all the tests were carried out in triplicates and the antibacterial activities were determined as mean diameter of inhibition zone (mm) produced by the extracts.

Sources of Brine Shrimp eggs and Brine Shrimp Lethality Bioassay

Natural sea water containing brine shrimp eggs were collected from natural sea water in Lagoon, Lagos. Natural sea water was poured into a hatching chamber, brine shrimp eggs were added at the closed section of the chamber. The open air section of the chamber was then exposed to fluorescent light for 48 hrs. This allows the eggs to mature and migrate through the opening to the exposed part of the dish. Three EDTA sample bottles of the same size were washed and sterilized before use. 20 mg of the sample was weighed and dissolved in 1 ml DMSO, few drops of *n*-hexane was added and then 1 ml of distilled water. This gives the stock solution [20 mg/ ml = 10,000 µg/ml]. Different concentration of the extracts were prepared in triplicates (1000, 100 and 10 µg/ml) by serial dilution. A control or blank was prepared by measuring5 ml of sea water into a test tube and adding 10 shrimp eggs. Ten brine shrimp larvae were carefully counted into each of the sample bottles and the volume of the sea water made up to 5 ml. The number of surviving larvae were counted and recorded after 24 hr. The data obtained were subjected to analysis (using Finney computer programme)[16] to determine the LC_{50}. The brine shrimp larvae have been used as target organism to detect bioactive compounds in plant extracts and toxicity to this crustacean has a good correlation with antitumour[17-20].

RESULTS AND DISCUSSION

The result of the preliminary phytochemical screening (**Table 1**) indicated that flavonoids and saponins were present in higher concentration in the extracts of *R. beninensis*, while alkaloids and tannins were in low concentration. These metabolites could be responsible for the antimicrobial properties of the extracts as claimed in the folkloric/traditional usage of *R. beninensis*. The inhibitory actions of the extracts on the tested strains are shown in **Table 2**. The result indicated a meaningful inhibition on the tested strains by the stem bark methanolic extracts of *R. beninensis* compared to the stem ethyl acetate extract. This is in direct conformity with the analysis of phytochemicals earlier carried out. The stem methanol and ethyl acetate extracts are active on all tested strains at different concentrations. In each case assayed, the standard antimicrobial drug (tetracycline) was more active than the extracts at the same dose. However, the zones of inhibition of the extracts were somehow comparable to that of the standard drug assayed in some cases. Isolation and purification may potentiate higher activity when the targeted compounds are fractionated out by means of column chromatography. The antimicrobial result got in this study confirmed the traditional use of various morphological parts of *R. beninensis* in traditional medicine. In related studies, alkaloids and flavonoids have been implicated as antimicrobial phytochemicals[21,22].

Table 1
Phytochemical analysis of the stem MEOH and EtOAc extracts of R. beninensis on dry weight basis (mg/kg)

Phytochemical Constituents	Methanol Extract	Ethyl acetate Extract
Alkaloids	1.50 ± 0.10[a]	1.10 ±0.20[b]
Flavonoids	6.24 ± 0.20[a]	6.10 ± 0.10[c]
Saponins	7.50 ± 0.10[c]	5.30 ± 0.15[a]
Tannins	1.32 ± 0.03[b]	2.10 ± 0.20[c]

Values are means ± standard deviation of triplicate of determination on dry weight basis. Values with the same superscript are not significantly different at P < 0.05.

Table 2
Sensitivity of the tested strains on the stem bark extracts of R. beninensis

Extracts	Concentration (mg/ml)	Diameters of zones of inhibition (DMZI, mm)			
		E. coli	*B. subtilis*	*S. typhii*	*S. aureus*
Methanol	50	12.10 ±0.10	15.10 ± 0.20	13.20 ± 0.15	10.15 ± 0.30
	20	10.24 ± 0.20	10.22 ± 0.10	7.10 ± 0.10	6.20 ± 0.10
Ethyl acetate	50	10.10 ± 0.30	12.22 ± 0.40	9.20 ± 0.20	9.65 ± 0.20
	20	8.55 ± 0.25	7.50 ± 0.10	8.15 ± 0.10	8.15 ± 0.10
Tetracycline	50	18.10 ± 0.30	19.20 ± 0.20	16.10 ± 0.10	13.20 ± 0.30
	20	12.10 ± 0.20	16.00 ± 0.10	12.20 ± 0.30	11.50 ± 0.40

Extracts having DMZI > 7.00 mm is considered active

The brine shrimp lethality assay detects substances that are cytotoxic enough to kill shrimp's larva on exposure to solution of the sample. A substance is cytotoxic if it inhibits vital metabolic processes or it causes disorder in living organisms resulting in perversion of behaviour or death[23,24]. The results of the brine shrimp lethality bioassay are described in **Table 3**. The results indicated that the cytotoxicity of the stem methanol and ethyl acetate extracts of *R. beninensis* increase with increase in concentration. At 100 µg/ml and 1000 µg/ml, none of the larvae survived in the stem methanol extract. Only 6 larvae survived in the methanol extract with LC_{50} of 3.20 at concentration level of 10 µg/ml. Similar feature was observed in the stem ethyl acetate extract, where 8 of the larvae survived at 10 µg/ml with LC_{50} of 2.50. Earlier study had traced cytotoxity to the presence of alkaloids, due to their bitter principles[25]. This result confirmed that stem methanol and ethyl acetate extracts of *R. beninensis* have significant lethality. Hence, it can be inferred that the stem methanol and ethyl acetate extracts of *R. beninensis* are toxic at the concentration analysed. At lower

concentration, and purification, the real toxicity of the phytochemical inherent in the stem methanol and ethyl acetate extracts of *R. beninensis* may be revealed. The various results obtained in this study confirm the folkloric usage of the various morphological parts of R. beninensis in traditional medicine.

Table 3
Brine shrimp cytotoxicity test of the stem methanol and ethyl acetate extracts of R. beninensis

Extracts	Dosage (mg/ml)	Survival		Mortality		LC_{50} (µg/ml)
Methanol	10	3	2	1	24	
	100	0	0	0	30	3.20
	1000	0	0	0	30	
Ethyl acetate	10	4	3	1	22	
	100	0	0	0	30	2.50
	1000	0	0	0	30	

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