

Full Length Research Paper

Biological activities and nutritional value of *Tapinanthus bangwensis* leaves

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Accepted 29 August, 2012

The cytotoxic and antioxidant properties of the ethanolic extract and fractions of the leaves of *Tapinanthus bangwensis* were evaluated. The investigation of their total phenol and flavonoid contents, proximate analysis and their mineral composition were also carried out. In the brine shrimp lethality assay, the ethyl acetate and dichloromethane fractions showed cytotoxic activity with LD₅₀ values of 11.22 and 15.84 µg/ml, respectively. The ethyl acetate and dichloromethane fractions exhibited significant cytotoxic effects towards HeLa cells with IC₅₀ values of 24.25 and 24.43 µg/ml respectively. In the DPPH assay, the ethyl acetate fraction showed the highest activity while the butanol fraction showed the highest activity in the metal chelating and the lipid peroxidation assays. The quantitative analysis gave the total phenol and flavonoid contents in the range of 13.38 to 668.65 mg gallic acid equivalent (GAE)/g and 0.53 to 34.52 mg quercetin equivalent (QE)/g, respectively. For the proximate analysis, the crude fibre, carbohydrate, protein, total ash and moisture content values were 58.03, 17.80, 7.79, 13.70 and 0.60%, respectively while iron occurred in the highest amount and sodium in the least in the mineral composition analysis. These results demonstrate the cytotoxic and antioxidant activities of *T. bangwensis* leaves and support the traditional use of the plant in cancer treatment.

Key words: *Tapinanthus bangwensis*, brineshrimp, HeLa, antioxidant.

INTRODUCTION

Africa is endowed with large resources of natural products and these have been used for centuries for the treatment of various diseases. More than 50% of all modern clinical drugs presently in use are of natural product origin and these have played a role in drug development programs in the pharmaceutical industry (Stuffness and Douros, 1982; Cordell, 1995).

Tapinanthus bangwensis (Engler and K. Krause) Danser (Loranthaceae) is a parasitic, woody shrub with a pendulous stem of up to 2 m long. It is found in the forest

region from Senegal to Cameroon and extends over the Congo basin to Zaire. Traditionally, the leaves are used in Ghana to treat guinea worm while in Nigeria, they are used to treat various ailments such as cancer and liver disorder. The plant is used with *Gardenia tricantha* D.C. (Rubiaceae) for the treatment of leprosy in Senegal (Burkill, 1995). Flavonoids, lectins, polypeptides, triterpenes and polyphenolic compounds have been reported in the plant (Duong et al., 2003). The presence of phlobotannins, alkaloids, anthraquinones as well as cardiac and steroidal glycosides have also been reported (Wahab et al., 2010). The study was carried out to investigate the *in vitro* cytotoxicity and antioxidant activities of the ethanolic extract and fractions of *T. bangwensis* as well as its total phenol and flavonoid

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contents, proximate analysis and mineral composition.

MATERIALS AND METHODS

Plant material

The leaves of *T. bangwensis* were collected from Ibadan, Oyo State, Nigeria in April, 2011. They were identified by Mr. Daramola at the Herbarium of the Department of Botany, University of Lagos where a voucher specimen (LUH 3856) was deposited.

Sample preparation

Air-dried and powdered leaves of *T. bangwensis* (500 g) were extracted with 2.5 L 80% ethanol at room temperature for 48 h. The extract was filtered and evaporated *in vacuo* at 30°C to yield a residue (11.97 g). This residue was suspended in water and partitioned successively with hexane (HF), dichloromethane (DF), ethyl acetate (EF) and n-butanol (BF) to yield 2.51, 2.15, 1.57 and 0.86 g residues, respectively.

In vitro cytotoxic activity

Brine shrimp lethality assay

The method of McLaughlin (1991) was used in this assay. The extract and fractions were weighed and used to prepare the stock solutions. 1 ml of each stock solution (50, 500, 5000 µg/ml) was put into a test tube already calibrated into 5 ml and made up to 5 ml with the filtered sea water to give overall concentrations of 10, 100 and 1000 µg/ml. Ten brine shrimp larvae were then placed in each of the test tubes. This was done in triplicates; the negative control consisted of sea water to which only 10 nauplii were added. Vincristine sulphate, an anticancer drug was used as a positive control.

After 24 h, the number of shrimps which survived were counted using a magnifying lens and recorded. All experimental assays were done in triplicates. The LD₅₀ was calculated using the Probit method (Wardlaw, 1985).

Cytotoxicity assay using HeLa cell line

Cervical (HeLa) cancer cells were seeded at 6,000 cells/well in 96-well plates and left to attach overnight at 37°C in a humidified incubator and 5% CO₂. The extract and fractions were resuspended in dimethyl sulfoxide (DMSO), sonicated for 15 min and complete RPMI-1640 added to reach concentrations of 25 to 100 µg/ml. The final concentration of DMSO did not exceed 0.25%. Cells were treated for 48 h after which the medium was replaced with 200 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) (0.5 mg/ml in RPMI 1640:10% FBS). After further 4 h incubation at 37°C, the MTT was removed and the purple formazan product dissolved in DMSO (Mossman, 1983; Koduru et al., 2007). The absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Cisplatin (10 and 100 µM) was used as positive control. All experimental assays were done in triplicates.

Antioxidant activity

DPPH radical scavenging capacity

Stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for

the determination of free radical scavenging activity of the extract and fractions (Blois, 1958). Five different concentrations of the extract/fractions (10 to 200 µg/ml) were added, in equal volumes, to the methanol solution of DPPH (100 µM). The reaction mixture was shaken thoroughly and left on the bench at room temperature for 30 min for complete reaction. The absorbance was measured at 517 nm using a spectrophotometer (T80 spectrometer, PG Instrument Ltd.). Quercetin was used as standard. All determinations were done in triplicate. The DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

Where, A is the absorbance of the control and B is the absorbance of the test sample.

Lipid peroxidation assay (LPO)

The method of Ohkawa et al. (1979) was used to assay the level of lipid peroxidation in the extract and fractions. Liver homogenate was used for the induction of lipid peroxidation mediated by FeSO₄ as pro-oxidant. The reaction mixture contained 1 ml of liver homogenate, 0.1 ml of potassium dihydrogen sulphate buffer (10 mM), 0.1 ml of FeSO₄.6H₂O (25 µM), 0.1 ml ascorbic acid (100 µM) and 1 ml of various concentrations of the extract/fractions (10 to 200 µg/ml). Distilled water (0.7 ml) was added to make up to 3 ml. Incubation was carried out at 37°C for 1 h after which the reaction mixture was treated with 1.0 ml thiobarbituric acid in HCl (0.8%) and 1 ml trichloroacetic acid (20%, pH 3.5). The solution was boiled in a water bath at 100°C for 30 min. The absorbance was measured at 532 nm. The assay was performed in triplicate and quercetin was used as standard. The percentage inhibition of lipid peroxidation was calculated using the following equation:

$$\% \text{ Inhibition of lipid peroxidation} = \frac{A - B}{A} \times 100$$

Where, A is the absorbance of the control reaction and B is the absorbance in the presence of the test sample.

Metal chelating activity

The metal chelating activity was determined using the method described by Dinnis et al. (1994). Distilled water (1.7 ml) was mixed with 50 µl of five concentrations of the extract and fractions ranging from 10 to 200 µg/ml followed by the addition of 50 µl of FeCl₂.4H₂O and the mixture was left to stand at room temperature for 1 min. 0.2 ml of 5 mM ferrozine was added to the mixture and incubated for 10 min. The absorbance was measured at 562 nm using a spectrophotometer (T80 spectrometer, PG Instrument Ltd.). Ethylenediaminetetraacetic acid (EDTA) was used as standard. All determinations were done in triplicate. The percentage inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

Where, A is the absorbance of the control reaction and B is the absorbance in the presence of the sample extract.

Determination of total phenolic and flavonoid contents

The total phenolic content of the ethanolic extract and fractions

Table 1. Cytotoxic activity of *T. bangwensis* crude extract and fractions.

Plant extract/ fraction	Brine shrimp lethality test LD ₅₀ (µg/ml)	HeLa cell line IC ₅₀ (µg/ml)
Crude	31.62	45.55
Hexane	89.13	75.16
Dichloromethane	15.84	24.25
Ethyl acetate	11.22	24.43
Butanol	1000	>200
Aqueous	446.68	>500
Vincristine sulphate	0.52	ND
Cisplatin	ND	0.45

ND, Not determined.

were determined by a modified Folin-Ciocalteu method (Wolfe et al., 2003). Briefly, 10 mg of the ethanolic extract/fraction was dissolved in 10 ml of methanol. The extract/fraction (0.5 ml) was mixed with 2.5 ml Folin-Ciocalteu reagent (diluted with water 1:1) and 2 ml (75 g/L) sodium carbonate. The tubes were vortexed for 15 s and allowed to stand for 30 min at 40°C for colour development. Absorbance of the mixture was measured at 760 nm using a spectrophotometer (T80 spectrometer, PG Instrument Ltd.). Gallic acid was used as the standard and the total phenolic content of the extract was expressed as milligram gallic acid equivalent (GAE) per gram of sample. Experiments were performed in triplicate.

Total flavonoid content was determined using the method of Ordonez et al. (2006). Quercetin was used as a standard. 1 ml of 2% AlCl₃ in ethanol was added to 1 ml of the extract. After 1 h incubation at room temperature, the absorbance was measured at 420 nm by using a spectrophotometer (T80 spectrometer, PG Instrument Ltd.). Experiments were performed in triplicate. Total flavonoid content was expressed as milligram quercetin equivalent (QE) per gram weight of sample.

Proximate analysis and determination of mineral content

The proximate composition (moisture, ash, crude fibre, carbohydrate and protein) of the leaves were determined using the standard methods of the Association of Official Analytical Chemists (AOAC, 1990).

For the mineral composition, calcium, sodium, potassium, magnesium, copper, zinc, iron and manganese were determined with an atomic absorption spectrometer (Analyst 200, Perkin Elmer, Waltham, MA, U.S.A.).

Statistical analysis

All values were expressed as means ± standard deviation (SD). Data were analysed by one way ANOVA and Student's t-tests.

RESULTS AND DISCUSSION

The brine shrimp lethality test is a simple bioassay used for the primary screening of crude extracts (CE) of plants (Meyer et al., 1982). It is an indication of cytotoxicity, anticancer, antiviral, pesticidal, and antimicrobial and other different pharmacological activities. In this investigation, the extract and fractions with LD₅₀ less than 50

µg/ml were considered active. The crude extract, ethyl acetate and dichloromethane fractions showed cytotoxic activity (Table 1). The cytotoxicity exhibited by the ethyl acetate fraction was however the most promising. Based on the results obtained from the brine shrimp lethality test, different dilutions of the extract and fractions (25, 50 and 100 µg/ml) were tested on human cervical cancer cell line for 24 h. Cisplatin was used as a positive control and it showed the highest inhibitory potency to the HeLa cells. The ethyl acetate and dichloromethane fractions exhibited significant cytotoxic effects towards HeLa cells with IC₅₀ values of 24.25 and 24.43 µg/ml, respectively (Table 1). The aqueous fraction (AF) showed the least inhibitory activity. Comparison of the results obtained in Table 1 revealed that there was correlation of the lethality effects of the extract/fractions between brine shrimps and the tested human cancer cell line since the hexane, dichloromethane and ethyl acetate fractions showed cytotoxic activity in both assays. This indicates that the mode of action for cytotoxicity of the extract and fractions is similar in brine shrimps and human cancer cell line. This is supported by the work of Anderson et al. (1991) which reported the correlation of the brine shrimp lethality test with cytotoxic and anti-tumour properties of human cancer cell lines *in vitro*.

Several methods have been used to determine the antioxidant capacity of plants. In this study, three methods were used to evaluate the antioxidant activity of *T. bangwensis* extract and fractions, namely, DPPH radical scavenging activity, lipid peroxidation and metal chelating assays. DPPH is one of the compounds that have a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). The DPPH assay has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants (Ruan et al., 2008). The ethyl acetate fraction showed the highest scavenging activity in the DPPH assay carried out in this work. In brief, the extract/fractions exhibited radical scavenging activity in the following order: ethyl acetate fraction (EAF) > BF > CE > DF > AF > HF (Figure 1). The activity of the ethyl acetate

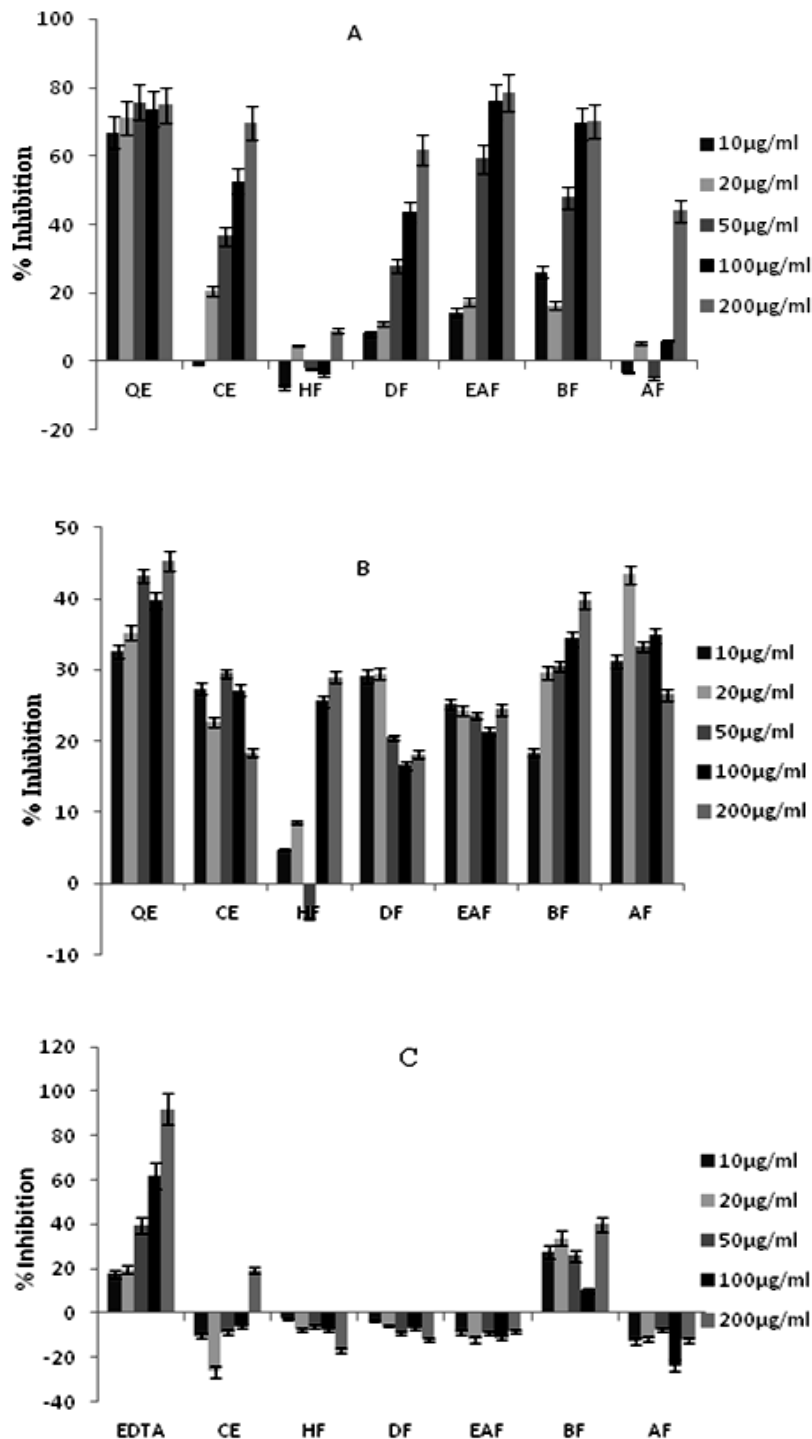


Figure 1. Antioxidant activity of the extract and fractions of *T. bangwensis* DPPH radical scavenging activity (A), lipid peroxidation (B) and metal chelating activity (C). Results represent the mean \pm standard deviation of triplicate determinations.

fraction may be attributed to the presence of strong radical scavenging compounds. The lipid peroxidation assay gives information on the scavenging of free radicals induced by oxidative stress which can initiate and propagate the lipid peroxidation cascade (Nuutila et

al., 2003). In this assay, the butanol fraction was the most active fraction and antioxidant activity was found to be in the order: BF > AF > DF > EAF > CE > HF. Iron stimulates lipid decomposing lipid hydroperoxides into peroxy and allyl radicals that can themselves reduce hydrogen

Table 2. Total phenolic and total flavonoids contents of *T. bangwensis*.

Extract/fraction	Total phenolic content (mg GAE/g dry wt)	Total flavonoid content (mg QE/g dry wt)
Crude extract (CE)	431.23 ± 0.017	10.64 ± 0.010
Hexane fraction (HF)	210.16 ± 0.050	23.69 ± 0.003
Dichloromethane fraction (DF)	219.08 ± 0.120	34.52 ± 0.017
Ethyl acetate fraction (EAF)	528.87 ± 0.030	12.07 ± 0.004
Butanol fraction (BF)	668.65 ± 0.180	8.25 ± 0.002
Aqueous fraction (AF)	13.38 ± 0.010	0.53 ± 0.003

Table 3. Proximate analysis of the leaves of *T. bangwensis*.

Composition (%)	Value
Moisture	0.60 ± 0.10
Ash	13.70 ± 0.02
Fibre	58.03 ± 0.002
Carbohydrate	17.80 ± 0.02
Protein	7.79 ± 0.03

Table 4. Mineral composition of the leaves of *T. bangwensis* (mg/g of dry matter).

Element	Composition
Iron	11.98
Calcium	10.30
Manganese	5.95
Copper	1.50
Zinc	1.10
Magnesium	0.75
Sodium	0.55
Potassium	0.25

and perpetuate the chain reaction of lipid peroxidation. The results show that the butanol fraction has the ability to protect against lipid peroxidation by scavenging the OH or the superoxide radicals by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself (Govindarajan et al., 2003). The ability to chelate/deactivate transition metals is an important mechanism of antioxidant activity. Chelating agents may serve as secondary antioxidants since they reduce redox potential, thereby stabilizing the oxidized forms of metal species. Chelating ability of plant extracts provide a strategy to avoid free-radical generation and iron-overload by chelation of metal ion (Robak and MaranKiewicz, 1995). In the metal chelating assay, only the butanol fraction interfered with the formation of ferrous and ferrozine complex and this suggests that secondary metabolites in the fraction have the ability to chelate iron before the formation of ferrozine.

The total phenolic and flavonoid contents were reported

as gallic acid and quercetin equivalent concentration (mg/ml), respectively. The results show that fractions contained phenolic compounds in the following order: BF > EAF > DF > HF > AQ and flavonoids in the following order: DF > HF > EAF > BF > AQ respectively (Table 2). The butanol fraction had the highest concentration of the total phenolics and also showed the highest antioxidant activity in the lipid peroxidation and metal chelating assays. These results suggest that the phenolic compounds may be responsible for the antioxidant activity observed as close correlation has been reported between the phenolic content and antioxidant activity of extracts obtained from various natural sources (Shahidi, 1997; Meyers et al., 2003; Skerget et al., 2005).

The proximate composition of *T. bangwensis* leaves is presented in Table 3. The low moisture content of the plant implies that it may have a long shelf-life since moisture content is an indication of water activity and can be used to measure the stability and the susceptibility to microbial contamination (Uraih and Izuagbe, 1990; Olutiola et al., 1991). The ash content is an indication of the mineral content of the sample. The carbohydrate (17.8%) and protein (7.79%) content of the leaves were low while the fibre content (58.03%) was high and this is a likely benefit derivable from the consumption of the plant as epidemiological studies suggest that increased fiber consumption may contribute to a reduction in the incidence of some diseases such as colon cancer, high blood pressure, coronary heart disease as well as digestive disorders (Dillard and German, 2000; UICC/WHO, 2005).

The importance of determining and evaluating the mineral content of plants is of utmost importance since significant human nutrition and health benefits can be obtained from an increase in the mineral contents of plant products (Grusak and Dellapenna, 1999). According to the results of the mineral content analysis in this study, iron and calcium were the most abundant elements (Table 4). Calcium is the major component of the bone and it also assists in tooth development (Brody, 1994). Iron is important for cell growth, oxygen transport as well as DNA synthesis. Low iron levels result in anemia which causes fatigue, shortness of breath, irritability, weight loss, dizziness and headaches (Shils et al., 1994).

However care must be taken as iron when taken in

excess, can become toxic and cause a myriad of health problems (Lynch, 1994).

Conclusion

The results from this present study confirm that *T. bangwensis* possesses cytotoxic and antioxidant activities which may be due to the phenolic compounds present in the extract. The leaves of the plant also contain an appreciable amount of fibre, carbohydrate, protein and mineral elements. These results support the traditional use of the plant in the treatment of cancer.

ACKNOWLEDGEMENT

The authors are grateful to the Africa Laser Centre for sponsoring the anticancer aspect of this work. The authors also thank Mr. Adenekan (Department of Biochemistry, University of Lagos) and Dr. M.O. Sofidiya (Department of Pharmacognosy, University of Lagos) for providing some of the chemicals used in this study.

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