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Full Length Research Paper

Antimicrobial activities of methanol and aqueous extracts of the stem of *Bryophyllum pinnatum* Kurz (Crassulaceae)

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The stem of *Bryophyllum pinnatum* (Crassulaceae), used in ethnomedicine for the treatment of various diseases, was screened for secondary metabolites and antimicrobial activity on *Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis*, *Candida albicans* and *Aspergillus niger*. Phytochemical analysis showed the presence of alkaloids, glycosides, proteins, carbohydrates, saponins, steroids, tannins and terpenoids in both the methanol and aqueous extracts. The antimicrobial activity result showed that the methanol extract significantly (P < 0.01) demonstrated antibacterial action against *B. subtilis* and *S. aureus* at 100, 50 and 25 mg/ml concentrations, using the agar diffusion technique. The aqueous extract also significantly (P < 0.01) showed antibacterial action against *S. typhi* and *B. subtilis* at the same concentrations. Both extracts did not demonstrate any antimicrobial activity against *P. aeruginosa, C. albicans* and *A. niger. S. aureus* showed the lowest minimum inhibitory concentration (MIC) of 6.29 mg/ml in the methanol extract, while *S. typhi* showed the highest MIC of 9.98 mg/ml in the aqueous extract (significant at P < 0.01). The results validate the use of *B. pinnatum* stem in ethnomedicine.

Key words: Antimicrobial, *Bryophyllum* stem extracts.

INTRODUCTION

The search for the therapeutic use of natural products is on the increase and this may be caused by the resistance of micro-organisms to many orthodox antibiotics. *Bryophyllum pinnatum* Kurz [syn. *Bryophyllum calycinum*, *Kalanchoe pinnata* Pers, common names: African – never – die, resurrection plant, life plant (Anonymous, 2005), miracle leaf, air plant (Wikipedia, 2009), Family Crassulaceae] is an erect, perennial, fleshy herb with a height of 60 to 120 cm. It is branched from the base, with opposite, simple or trifoliolate, petiolate leaves. The glabrous, thick, fleshy leaf is about 10 cm long and 5 to 6 cm broad, obovate to obovate – orbicular, coarsely crenate and sometimes bears bulbils in the axils. The drooping flowers occur in lax panicles with inflated, tubular, 4-lobed calyx that is about 3cm long. The calyx lobes are triangular and very acute with greenish yellow and purplish base. The tubular 4-lobed, gamopetalous corolla are contracted above the base and a little longer than the calyx. The corolla lobes are ovate – lanceolate, abruptly acute – acuminate and reddish purple at the upper part. Plantlets may grow along the notches of the leaf margins and can develop while still attached to the plant or when detached. The plant is native to Madagascar, introduced to and now naturalized in many parts of Tropical Africa, Asia and South America (Hutchinson and Dalziel, 1954).

The plant can be used in treating high blood pressure, stroke, convulsion, pain, epilepsy, candidiasis, bladder infection and also as an anti-poison (Anonymous, 2005). In traditional medicine, *B. pinnatum* has been used to treat infections, rheumatism, inflammation, hypertension and kidney stones (Wikipedia, 2009). The pounded fresh

material is applied as a poultice for sprains, boils, abscess, eczema, infections, burns, carbuncle and erysipelas. Okwu and Josiah (2006) reported that the availability of ascorbic acid in *B. pinnatum* provides the biochemical basis for the ethnomedical use of the plant extract for the treatment and prevention of infections, cold and other diseases like prostrate cancer. Glycosides, steroids, flavonoids, bufadienolides, organic acids, alkaloids, tannins, phenolic compounds, gums, mucilages, lignin, etc., have been detected in the leaves of *B. pinnatum* (Marriage and Wilson, 1971; Kamboj and Saluja, 2010). Also, bryophillin A, B and C, a potent cytotoxic bufadienolide orthoacetate was found in the leaf extract (Yamagishi et al., 1989). Bryophillin C show insecticidal properties (Supratman et al., 2000). *K. pinnata* contains bufadienolide cardiac glycosides which can cause cardiac poisoning, particularly in grazing animals (McKenzie and Dunster, 1986; McKenzie et al., 1987). *Kalanchoe* extracts also have immunosuppressive effects (Lans, 2006).

The flavonoids, polyphenols, triterpenoids and other chemical constituents of the plant were speculated to account for the antinociceptive, anti-inflammatory, anti hypertensive and antidiabetic properties observed in the aqueous leaf extracts (Ojewole, 2005). Mudi and Ibrahim (2008) reported that the n-hexane fraction of *B. pinnatum* leaves showed antibacterial activity against *Staphylococcus aureus, Klebsiella pneumoniae* and *Salmonella typhi*, while the ethyl acetate soluble fraction showed mild activity against *Escherichia coli, S. aureus* and *S. typhi.* Broad spectrum antibacterial and antifungal activities were detected in the crude leaf extract of *B. pinnatum* (Aquil and Ahmad, 2003). The aqueous leaf extract of *B. pinnatum* had a neurosedative and muscle relaxant activities and produced a depressant action on the central nervous system of mice (Yemitan and Salahdeen, 2005). This depressant action was attributed to bufadienolide and other water soluble constituents in the extract (Salahdeen and Yemitan, 2006). Igwe and Akunyili (2005) reported that the aqueous leaf extract of *B. pinnatum* has a strong analgesic potency comparable in a time and dose – dependent manner to a non steroidal anti-inflammatory drug.

Most of these investigations were centered on the leaf extract of *B. pinnatum.* However, not much work has been carried out on the stem of this highly medicinal plant. The objective of this work therefore was to investigate the antimicrobial activities of the stem of *B. pinnatum.*

MATERIALS AND METHODS

Collection of plant material and phytochemical screening

The stem of *B. pinnatum* Kurz was collected at Nsukka, Enugu State and authenticated by Mr. A. Ozioko, a Taxonomist of the Bioresearch Development and Conservative Programme Centre in Nsukka, Enugu State. A voucher specimen (U.N.H. No. 1/15) was deposited at the Herbarium in Botany Department, University of Nigeria, Nsukka, Nigeria. The stem was air dried and pulverized. 300 g of the powdered stem was macerated separately with 1.5 L of methanol and 1.5 L of distilled water and both were concentrated to dryness with a vacuum pump. Phytochemical screening was carried out on the methanol and the aqueous extracts to determine the secondary metabolites content (Harborne, 1973; Trease and Evans, 1983).

Test for antimicrobial activity

Antimicrobial tests were carried out on the methanol and aqueous extracts using the agar diffusion method (Pelczar et al., 1993). 200 mg of each extract was dissolved in 2 ml of dimethyl sulphoxide (DMSO) to obtain 100 mg/ml concentration. Further dilutions of the stock solution were made using a two-fold serial dilution technique, to give 50, 25, 12.5 and 6.25 mg/ml concentrations. This same dilution was carried out on gentamicin (as a reference drug) to obtain 100, 50, 25, 12.5 and 6.25 µg/ml, concentrations. Nutrient agar medium was used to test the following isolates: *S. typhi, P. aeruginosa, S. aureus, B. subtilis, C. albicans* and *A. niger*. These organisms were clinical isolates obtained from the University of Nigeria Medical Centre, Nsukka. They were identified at the Department of Microbiology, University of Nigeria, Nsukka. *S. typhi, P. aeruginosa, S. aureus* and *B. subtilis* were maintained on blood agar slants at 4°C before use, while *C. albicans* and *A. niger* (fungi) were preserved on Sabourand's dextrose agar (Oxoid) slants at 4°C, prior to use. 20 ml of sterile solidified Mueller-Hinton nutrient agar was poured into a sterile Petri dish and seeded with 0.1 ml of standardized broth culture of the test micro-organism (1.0×10^7 cfu/ml). This was carried out for all the test micro-organisms. Five equidistant wells were made in each of the plates with a sterile 6.0 mm diameter cork borer. Using a sterile dropper, 0.3 ml of each of 100, 50, 25, 12.5 and 6.25 mg/ml concentration of the extracts were dispensed into each corresponding well, made in the plates. A well containing gentamicin was made in each of the plates seeded with the bacteria, while the plates seeded with fungi had ketoconazone in the well, as a reference drug. The plates were allowed to stand for 1 h for the prediffusion of the extract to occur. The plates with bacteria were incubated at 37°C for 24 h while those with fungi were incubated at 25°C for 48 h. They were observed and the presence of zones of inhibition around the wells were measured and taken as an indication of antimicrobial activity (Alade and Irobi, 1993). The experiment was carried out in two replicates and the mean for each organism was determined.

For the determination of MIC, methanol and aqueous extracts, as well as gentamicin (as a reference drug) were used on the susceptible micro-organisms. The agar diffusion method (Pelczar et al., 1993) was adopted. 200 mg of each extract was dissolved in 2 ml of DMSO to obtain 100 mg/ml concentration. Two-fold serial dilution was made to obtain 50, 25, 12.5 and 6.25 mg/ml concentrations. The same two-fold dilutions of 100 µg/ml gentamicin were made to obtain 50, 25, 12.5 and 6.25 µg/ml concentrations. These concentrations were put into wells bored in the seeded agar plates containing the susceptible micro-organisms, as above. The experiment was carried out in two replicates. The agar plates were placed in an incubator at 37°C for 24 h, after which the mean diameter of the zone of inhibition was measured. The graph of the square of the inhibition zone diameter was plotted against log concentration for each micro-organism. A regression line was drawn through the points. From these graphs, the representative MIC values were determined as the antilogarithm of the intercept on the logarithm of concentration axis. Analysis of variance (ANOVA) was determined on the data obtained, while the multiple comparisons were carried out between treatment means using Duncan's multiple range tests at $P< 0.05$ confidence level (Edafiogho, 2006).

Table 1. Result of the phytochemical analyses of the methanol and aqueous extracts of *Bryophyllum pinnatum* stem.

Test	Methanol extract	Aqueous extract
Acidic compounds	-	-
Alkaloids	+++	++
Carbohydrates	+++	+++
Fats and Oil	-	-
Flavonoids	++	-
Glycosides	+++	++
Proteins	++++	+++
Resins	++	-
Saponins	+	++++
Steroids	++++	+
Tannins	+	+
Terpenoids	++++	+
Reducing sugar	+++	++

-, Absent; + present in low concentration; ++ present in moderate concentration; +++ present in high concentration; ++++ present in very high concentration.

RESULTS

The methanol stem extract revealed the presence of high concentrations of alkaloids, carbohydrates, glycosides, proteins, steroids, terpenoids and reducing sugar, while the aqueous extract showed carbohydrates, proteins and saponins in high concentration (Table 1). Acidic compounds, fats and oils were not detected in both extracts. The results of the antimicrobial activity showed that the methanol stem extract significantly ($P< 0.01$) demonstrated antibacterial activities against *B. subtilis* and *S. aureus*, while the aqueous stem extract significantly ($P< 0.01$) showed antibacterial activities against *S. typhi* and *B. subtilis*, both at a concentration of 25 mg/ml and above (Table 2). Also, the methanol and the aqueous extracts showed the least antibacterial activities at a concentration of 12.5 mg/ml for *S. aureus* and *B. subtilis*, respectively. However, both extracts did not show any antimicrobial activity against *P. aeruginosa, C. albicans* and *A. niger*. For both extracts, 100 mg/ml was significantly ($P< 0.01$) the most effective against all the bacteria used. However, for the methanol extract, 100 mg/ml was not significantly different from 50 mg/ml, as regards *S. aureus.* The lower the concentration, the lower the effectiveness to the extent that 6.25 mg/ml concentration exhibited no activity against any micro-organism. Comparatively, gentamicin (the standard drug) showed significantly ($P< 0.01$) higher antibacterial activities against *B. subtilis, S. aureus* and *S. typhi* at a concentration of 12.5 µg/ml and above.

For MIC, the antibacterial activities of the methanol extract were significantly ($P< 0.01$) different from those of the aqueous extract and gentamicin (Table 3). *S. aureus* showed the lowest MIC in the methanol extract, while *S. typhi* demonstrated the highest MIC in the aqueous extract. However, for gentamicin, the reverse was the case for MIC. The methanol extract inhibited *B. subtilis* more than the aqueous extract. These effects were significant at $P<0.01$.

DISCUSSION

In this investigation, the methanol and the aqueous extracts of *B. pinnatum* stem demonstrated antibacterial activity against three out of the six micro-organisms used for the test. This antibacterial activity may be attributed to the alkaloids, glycosides, steroids, terpenoids, saponins, flavonoids and perhaps resins, since these secondary metabolites were detected in the extracts. This is similar to the work of Marriage and Wilson (1971), who reported that a number of active compounds, including flavonoids, glycosides, steroids, bufadienolides and organic acids have been identified in *B. pinnatum* leaves. These constituents are known to demonstrate activity against micro-organisms. The extracts used in this work exhibited antibacterial activity at a concentration of 12.5/25 mg/ml and above. The results are similar to the work of Akinpelu (2000) who found that the methanol extract of the leaves of *B. pinnatum* inhibited the growth of five out of eight bacteria used, at a concentration of 25 mg/ml. The aqueous and alcoholic extracts of the leaves of *B. pinnatum* and *K. crenata* demonstrated antimicrobial activities against *E. coli, P. aeruginosa, K. pneumoniae, Shigella flexneri, S. paratyphi*, *Citrobacter* spp., *S. aureus, E. faecalis* and *B. subtilis* (Akinsulire et al., 2007). In this work, the aqueous and methanol extracts of the stem did not show any antimicrobial activity against *P. aeruginosa, C. albicans* and *A. niger*. Similarly, Akinsulire et al. (2007) found that *C. albicans* was not susceptible to

Table 2. Mean inhibitory zone diameter (mm) of different concentrations of methanol and aqueous extracts of *Bryophyllum pinnatum* stem and gentamicin on the test micro-organisms.

Micro-organism	Methanol extract					Aqueous extract					Gentamicin				
	Concentration (mg/ml)					Concentration (mg/ml)					Concentration (µg/ml)				
	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25
S. typhi	-	-	-	-	-	18.0 ± 1.0^{a}	14.0 ± 0.0^{i}	9.5 ± 0.5^{o}	-	-	29.5 ± 0.5^{b}	28.0 ± 0.0^{j}	21.0 ± 0.0^{p}	17.5 ± 0.5^{k}	-
B. subtilis	25.0 ± 0.0^{c}	21.5 ±0.5^{g}	20.0 ± 0.0^{q}	-	-	24.0 ± 1.0^{c}	20.5 ± 0.5^{g}	15.5 ± 0.5^{t}	13.0 ± 1.0^{t}	-	37.5 ± 0.5^{d}	32.0 ± 0.0^{h}	24.5 ± 0.5^{s}	23.0 ± 0.0^{u}	-
S. aureus	22.5 ± 0.5^{e}	21.0 ± 0.0^{e}	17.0 ± 1.0^{m}	11.0 ± 1.0^{v}	-	-	-	-	-	-	34.0 ± 0.5^{f}	31.0 ± 0.0^{l}	30.0 ± 0.0^{l}	21.5 ± 0.5^{w}	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. niger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- , no activity. Values represent means ± standard error. Means followed by the same letter(s) within the same row and column are not significantly different.

Table 3. Minimum inhibitory concentrations (MIC) of gentamicin, methanol and aqueous extracts of *Bryophyllum pinnatum* stem on the test micro-organisms.

Micro-organism	Methanol extract (mg/ml)	Aqueous extract (mg/ml)	Gentamicin (µg/ml)
Salmonella typhi	-	9.98 ± 0.01^{c}	5.25 ± 0.01^{e}
Bacillus subtilis	8.42 ± 0.01^{a}	6.42 ± 0.1^{d}	5.83 ± 0.01^{f}
Staphylococcus aureus	6.29 ± 0.01^{b}	-	5.97 ± 0.01^{g}

-, No activity. Values represent means ± standard error. Means followed by the same letter(s) within the same row and column are not significantly different.

the leaf extracts they obtained from the traditional method. In this investigation, the methanol extract of the stem of *B. pinnatum* was more active than the aqueous extract. This is also similar to the work of Akinsulire et al. (2007) who reported that of all the extracts of *B. pinnatum* leaf, the methanol extract was the most active. Aquil and Ahmad (2003) reported that the ethanolic extract of *B. pinnatum* leaves had broad-spectrum antimicrobial activity. Ofokansi et al. (2005) reported that *B. pinnatum* leaf is effective in the treatment of typhoid fever and other bacterial infections, particularly those caused by *S. aureus, E. coli*, *B. subtilis*, *P. aeruginosa, Klebsiella aerogenes*, *K. pneumoniae* and *S. typhi.* These investigations supported the use of *B. pinnatum* in treating the placenta and navel of a new born baby, which heal fast and prevent infections (Okwu, 2003).

Taylor (2010) explained that the traditional use of *B. pinnatum* for the treatment of internal and external infections is supported by the fact that the leaves have antibacterial, antiviral and antifungal activities. An aqueous extract of the leaves administered topically and internally has been shown to prevent and treat leishmaniasis in both human and animals. In addition, the traditional uses of the plant for upper respiratory conditions and cough might be explained by the report that shows that the leaf juice has potent anti-histamine and anti-allergic activities. *In vivo* studies with rats and guinea pigs showed that the leaf juice was able to protect against chemically induced anaphylactic reactions and death by selectively blocking histamine receptors in the lungs. Another *in vivo* study showed that the leaf extract protected mice from ulcer-inducers such as stress, aspirin, ethanol and histamine, thus validating the traditional use of the plant for gastric ulcers. Other *in vivo* investigations confirmed that the leaf extract can reduce fever, provides anti-inflammatory, pain-relieving and muscle-relaxant

effects. Its anti-inflammatory effects have been partially attributed to the immunomodulatory and immune suppressant effects. Animal studies have shown that the leaf extract possesses sedative and central nervous system depressant action. These effects were attributed partially to the ability to increase the levels of a neuro transmitter, Gamma aminobutyric acid (GABA), in the brain (Taylor, 2010). Results obtained from animal studies indicate that the aqueous and methanol extracts of *B. pinnatum* leaf possess antihypertensive properties. This lends credence to the folkloric use of the herb in the management of hypertension by some Yoruba people of Western Nigeria (Ojewole, 2002).

In this investigation, 100 mg/ml was the most effective against the susceptible bacteria used and the lower the concentration, the lower the effectiveness, to the extent that 6.25 mg/ml showed no activity against any micro-organism. This is similar to the work of Nwadinigwe (2009), who reported that the 150 mg/ml concentration of the ethyl acetate fraction of *Emilia sonchifolia* was the most effective against the bacteria used. Also, the lower the concentration, the lower the effectiveness to the extent that 9.375 and 4.688 mg/ml showed no activity against any micro-organism. In this work, *S. aureus* showed the lowest MIC value, while *S. typhi* demonstrated the highest MIC. This is somehow similar to the work of Nwadinigwe (2009) who reported that *B. subtilis* exhibited the lowest MIC value, while *S. typhi* showed the highest MIC for the ethyl acetate fraction of *E. sonchifolia.*

In conclusion, *B. pinnatum* stem has the potential to be used as an antimicrobial agent, just like the leaves. However, further laboratory and clinical studies are required to determine its potency and safety.

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