

Full Length Research Paper

High performance liquid chromatography-diode array detection (HPLC-DAD) profiling, antioxidant and anti-proliferative activities of ethanol leaf extract of *Berlinia grandiflora* (Vahl) Hutch. & Dalziel

Aliyu Adamu*, Kevwe Benefit Esievo, Grace Ugbabe, Samuel Ehiabhi Okhale and Henry Omoregie Egharevba

National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

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Berlinia grandiflora is used for managing numerous ailments including tumor in South-west, Nigeria. The phytochemical contents, antioxidant and antiproliferative activities of *B. grandiflora* leaf were determined in this study. The absolute ethanol extract was evaluated by high performance liquid chromatography-diode array detection (HPLC-DAD). The antioxidant activity was carried out by standard analytical methods. The antiproliferative activity was done using growth inhibition activity of *Sorghum bicolor* seed radicle with methotrexate as the reference drug. The presence of some phytochemicals known to have antioxidant properties and exert anticancer effects is revealed in this study. The HPLC analysis revealed the presence of betulinic acid, ferulic acid, rutin and caffeic acid. However, ferulic acid, rutin, and caffeic acid were identified for the first time in *B. grandiflora*. Free radical scavenging activity, reducing power capacity and nitric oxide inhibitory activity were exhibited by the extract, with IC_{50} values of 88.3, 7.4 and 99 $\mu\text{g/mL}$, respectively. While ascorbic acid, the reference compound, used in this study has IC_{50} values of 0.7, 3.4 and 7.4 $\mu\text{g/mL}$, respectively. The total phenol content expressed in gallic acid equivalent (GAE) was 75 mg/g. A dose-dependent antiproliferative activity was observed with IC_{50} values of 0.50, 0.59 and 0.29 $\mu\text{g/mL}$ after 48, 72 and 96 h of treatment, respectively. This result justifies the folklore use of the plant for managing cancer in Nigeria and the plant's potential as a new phyto-chemo-therapy.

Key words: Antiproliferative, antioxidant, phytochemicals, cancer, *Berlinia grandiflora*, betulinic acid.

INTRODUCTION

Berlinia grandiflora (Vahl) Hutch. & Dalziel is a medicinal plant which is widely distributed across African countries; it belongs to the family Fabaceae. The genus *Berlinia* has about 20 species which are found in eastern, western and southern Africa, especially in countries like Guinea, Mali, Nigeria, Central African Republic and DR Congo

(Hutchison and Dalziel, 1963). Locally in Nigeria, *B. grandiflora* is called "Apado" by the Yoruba tribe and "Ububa" by the Igbo ethnic groups (Asuzu et al., 1993). The medicinal value of *B. grandiflora* is well known to herbalists and traditional medicine practitioners in South west of Nigeria. The plant is used for the treatment of

*Corresponding author. E-mail: adamualiyu246@gmail.com. Tel: +2348076978343.

several disease conditions in ethnomedicine. The bark sap is used for dressing wound and sores, while the bark decoction is used for managing haemorrhoids and liver problems. The bark extract is also used to ease labor pain during childbirth and for treating gastrointestinal disorder (Josephs et al., 2012). Leafy twigs decoction is used to ameliorate fever conditions and to combat vomiting and nausea. The leaf decoction is drunk as a tonic and used to improve blood circulation (Ode et al., 2013). The leaf extract is used to manage cancer in south-western Nigeria, as a vermifuge, and for treating diarrhea and diabetes (Soladoye et al., 2010; Ode et al., 2013). Previous researchers have reported the phytochemical analysis, analgesic activity (Asuzu et al., 1993), antihelminthic activity (Enwerem et al., 2001), antioxidant activity, antimicrobial activity (Josephs et al., 2012; Ode et al., 2013) and the anti-diabetic activity (Duru et al., 2014) of *B. grandiflora*. Also, the cytotoxicity of chemical compounds in the seed of the plant has been reported by Duru et al. (2014).

The leaves and stem barks of *B. grandiflora* have been reported to possess antimicrobial activity against a wide range of bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Alcaligenes faecalis*, *Serratia marcescens*, *Enterobacter bacteriaerogenes*, *Kliebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus vulgaris* (Josephs et al., 2012). The antimicrobial property of *B. grandiflora* is attributed to betulinic acid (Enwerem et al., 2001). The major compounds in the aqueous extract of *B. grandiflora* seeds are: α -methylmannofuranocide (21%), Pentadecanoic acid (15%), 9-octadecanoic acid (31%), Octadecanoic acid (17%) and 9-octadecinamide (16%). These compounds may be responsible for the acute toxicity of the seed of *B. grandiflora* in animals (Duru et al., 2014). The methanol extract of the leaf also shows acute toxicity in mice (Josephs et al., 2012). Methanol extract of the leaf and stem barks of *B. grandiflora* contain tannins, alkaloids, flavonoids, starch and glycosides (Asuzu et al., 1993; Ode et al., 2013). Similarly, the aqueous extract of the seeds was reported to contain alkaloids, saponins, tannins, phenol, flavonoids and starch, while anthraquinones, phenols, steroids and cyanogenic glycoside were absent (Josephs et al., 2012).

Although *B. grandiflora* is used in ethnomedicine as an anti-tumor agent, no scientific report of the antiproliferative activity of the leaf of the plant exists in literature. Therefore, the aim of this study is to scientifically evaluate the antiproliferative activity and antioxidant properties of the ethanol extract of the plant leaf with a view to providing scientific information to justify its use for managing cancer and tumors.

MATERIALS AND METHODS

Sampling, sample preparation and extraction

Fresh leaves of *B. grandiflora* were harvested in May 2016 from

Chaza, Suleja Local Government Area, Niger State, Nigeria and were identified by an expert at the Herbarium of the National Institute for Pharmaceutical Research and Development, Nigeria. Herbarium specimen was deposited and voucher number NIPRD/H/6966 was assigned. The fresh leaves were air dried at room temperature for 10 days after which they were crushed with the aid of a mortar and pestle. 100 g of the pulverized samples was macerated with 400 mL of absolute ethanol in an airtight container and placed on a mechanical shaker for 24 h. The resultant mixture was vacuum filtered using filter paper (Whatman No. 1) and concentrated using a rotary evaporator. The concentrate was then dried on a water bath to produce the dry ethanol extract.

Phytochemical analysis

Screening of phytochemical in the ethanol extract of the leaf of *B. grandiflora* was done to determine the presence of tannins, phenols, alkaloids, anthraquinones, saponins, terpenes, steroids, and flavonoids, which are secondary metabolites, using standard methods described by early workers (Evans, 2005; Sofowora, 1993; Trease and Evans, 1989).

High-performance liquid chromatography analysis

Chromatographic analysis was done with the aid of Shimadzu HPLC system comprising Ultra-Fast LC-20AB prominence equipped with SIL- 20AC autosampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector (UV-DAD); column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5 μ m and dimensions (150 \times 4.6 mm). Method reported by Okhale et al. (2017) was used with some modifications. The chromatographic conditions included mobile phase solvent A: HPLC grade water with 0.2% v/v formic acid and solvent B: HPLC grade acetonitrile; mode: isocratic; the flow rate is 0.6 mL/min; and injection volume of 1.0 μ L of 10 mg/mL solution of ethanol extracts of the leaf of *B. grandiflora*; detection was at UV 254 nm wavelength. Reference standards betulinic acid, rutin, quercetin, ferulic acid, caffeic acid, and apigenin were analyzed separately under the same conditions as the extract. The HPLC operating conditions were programmed to give the following: solvent B: 20% and column oven temperature of 40°C. The total run time was 22 min.

Antioxidant activity

Free radical scavenging assays

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a relatively stable free radical, which is commonly employed to determine the radical scavenging capacity of antioxidant compounds. This method relies on the reduction of DPPH in an alcohol solution in the presence of a hydrogen-donating species (antioxidant compounds) due to the formation of the non-radical form DPPH-H. This reaction produces a color change from purple to yellow, which is measured by a spectrophotometer. The disappearance of the purple color is monitored at 517 nm. The reaction mixture comprises 1.0 mL of 0.3 mM DPPH in methanol, 1.0 mL of the different concentrations of the extract (250, 125, 62.5, 31.25 and 15.625 μ g/mL) and 1.0 mL of methanol. It was incubated for 10 min in the dark, and then the absorbance was measured at 517 nm. In this assay, the positive control was ascorbic acid (Ode et al., 2013). The percentage of antioxidant activity can be calculated using the formula:

$$\text{Antioxidant activity (\%)} = 100 - \left(\frac{A_0 - A_1}{A_0} \times 100 \right)$$

where A_0 = absorbance of control, A_1 = absorbance of sample.

Nitric oxide inhibition

Nitric oxide produced from sodium nitroprusside is measured by the Greiss reaction. Briefly, sodium nitroprusside (10 mM) in phosphate-buffered saline (pH 7.4) was mixed with different concentrations of the extract (250, 125, 62.5, 31.25 and 15.625 µg/mL) dissolved in phosphate-buffered saline in a test tube and incubated at 25°C for 180 min in the dark. 1 mL of the mixture was reacted with 1 mL of Greiss reagent (1% sulphanilamide, 2% H₃PO₄ and 0.1% naphthylethylenediamine di-hydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 550 nm. Inhibition of nitrite formation by the plant extract was calculated relative to ascorbic acid which was used as the standard antioxidant drug. The IC₅₀ value was determined using graph pad.

Reducing power capacity

The determination of the reducing power was conducted according to the method described by Kim et al. (2015). Briefly, 2.5 mL of 250, 125, 62.5, 31.25 and 15.625 µg/mL concentrations of extracts were mixed with 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of 30mM potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. 2.5 mL of 600 mM of trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was mixed with 2.5 mL distilled water and 0.5 mL of 6mM ferric chloride, and absorbance was measured at 700 nm with a UV-visible spectrophotometer. Ascorbic acid was used as a standard. Results were presented as IC₅₀.

Determination of total phenolic content

The total phenolic content in the plant extracts was determined using the method described by Singleton et al. (1999). The reaction mixture was prepared by mixing 0.5 mL of extract, 2.5 mL of 10% Folin-Ciocalteu's reagent and 2.0 mL of 7.5% Na₂CO₃. Blank was also prepared to contain 0.5 mL ethanol, 2.5 mL of 10% Folin-Ciocalteu's reagent and 2.0 mL of 7.5% of Na₂CO₃. Samples were allowed to stand for 30 min. The absorbance was measured using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean values of absorbance were obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration curve was constructed. The phenolic content of the extract was determined from the calibration curve and expressed in terms of gallic acid equivalent (GAE mg/g of extract).

Determination of antiproliferative activity

Seed viability test

The seeds of *Sorghum bicolor* (Guinea corn) used in this experiment were purchased from IBB market, Suleja LGA, Niger State and were duly identified and authenticated. The seeds were subjected to a viability test by transferring the seeds into a beaker containing water. The floating seeds were discarded while the fully submerged seeds were cleaned, dried and used for the assay (Okhale et al., 2017).

Determination of growth inhibitory effect

The growth inhibition ability of the ethanol extracts of the leaves of *B. grandiflora* on the radicle length of the growing seeds of *S. bicolor* was determined using the modified methods of Ayinde et al.

(2010) and Chinedu et al. (2014). Briefly, 4 mg/mL stock solution of the extract was prepared and serially diluted into two solutions; 1 and 0.5 mg/mL. The test seeds were separated into six groups; four groups for the four different test solutions of *B. grandiflora* extract and treated with the test solution accordingly; the other two groups for positive control and control groups. Petri dishes were layered with cotton wool and filter paper (Whatman No. 1) and twenty-four (24) seeds of pre-treated *S. bicolor* (which has been soaked in the extract for 24 h) were placed in each of the Petri dishes. Each specific Petri dish with seeds received 15 mL of the particular concentration of the test solution used for pre-treatment (the seeds in first Petri dish were treated with extract concentration of 0.5 mg/mL, seeds in the second Petri dish received 1 mg/mL, the third received 2 mg/mL and the fourth received 4 mg/mL). 15 mL of 0.05 mg/mL of methotrexate was used as the positive control while the control seeds were treated with 15 mL of distilled water only. The experiments were carried out in triplicates and the seeds were incubated in a dark room and observed for growth after 24 h. The mean lengths (mm) of radical emerging from the seeds were measured after 48, 72 and 96 h. The percentage inhibition and percentage growth after 48, 72 and 92 h of treatment with extracts and the positive control methotrexate at 0.05 mg/mL relative to control were calculated as follows.

$$\% \text{ Inhibition} = \frac{L_c - L_t}{L_c} \times 100$$

where L_c is the mean radical length of control and L_t is the mean radical length of extracts.

$$\% \text{ growth} = 100 - \% \text{ Inhibition}$$

Statistical analysis

The data were subjected to statistical analysis using Microsoft Office Excel® software. Graph pad prism was used to calculate the IC₅₀ value.

RESULTS AND DISCUSSION

Phytochemical constituents

The extraction yield of the dried leaves of *B. grandiflora* was 7.28%. Phytochemical screening of the extract reveals the presence of alkaloids, saponins, terpenes, steroids, flavonoid, phenols and tannins. However, anthraquinones were not detected in the test sample. Previous studies have shown that *B. grandiflora* leaf contains tannins, alkaloids, flavonoids, glycosides, saponins, phenols while anthraquinones and carbohydrates were absent (Ode et al., 2013). The result of the phytochemical analysis in the present study is also consistent with the report of Asuzu et al. (1993) and Duru et al. (2014). The therapeutic property of plants is widely due to the presence of phytochemicals in them. Previous studies have shown that phytochemicals possess several pharmacological activities such as; anti-inflammatory activity, anti-atherosclerotic activity, anti-tumor activity, anti-mutagenic activity, anti-cancer activity, anti-bacterial activity, anti-viral activity to mention a few (Adamu et al., 2017). Specifically, phenolics which comprise flavonoids,

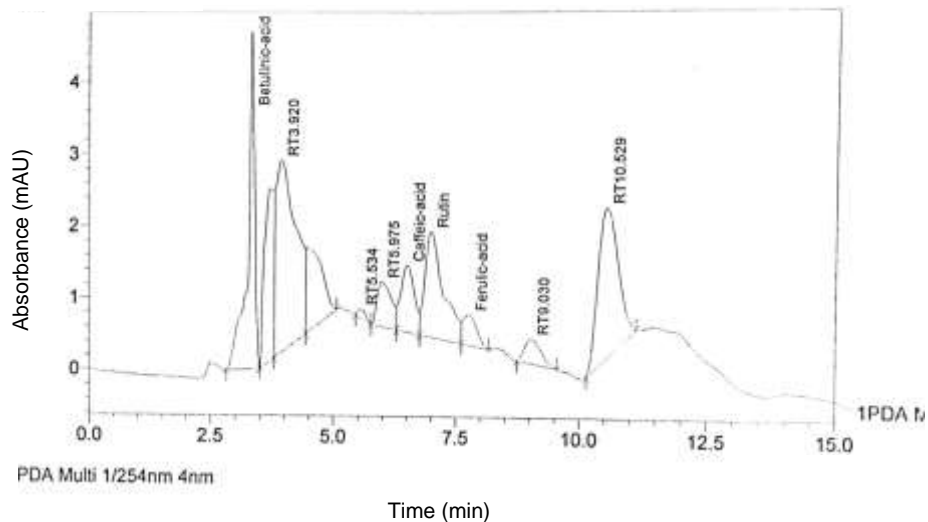


Figure 1. HPLC-DAD Chromatogram of ethanol extract of *Berlinia grandiflora*

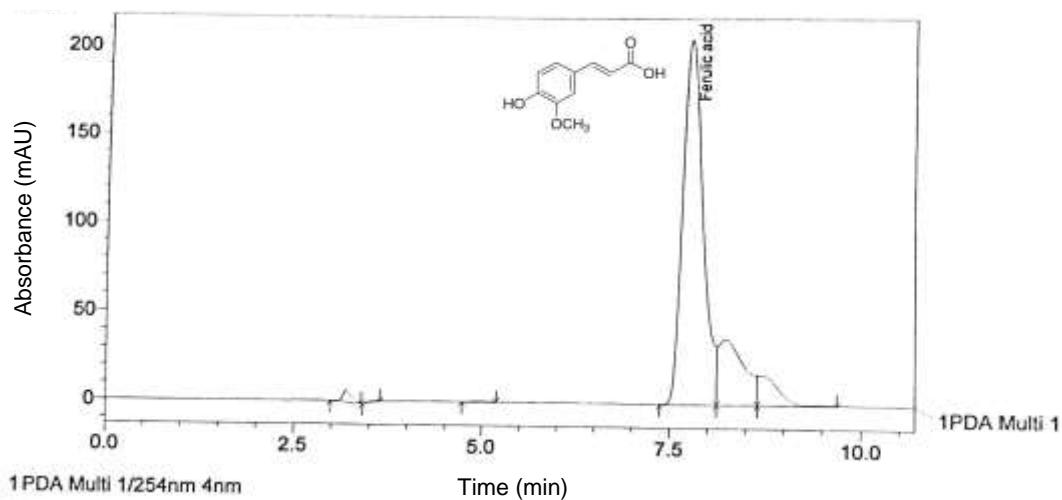


Figure 2. HPLC-DAD Chromatogram of Ferulic acid.

tannins and phenols represent phytochemicals linked to antioxidant properties of plant. Phenolic compound has been proven to exhibit preventive and therapeutic effects against diseases where oxidative stress has been implicated, including cardiovascular diseases, cancer, neurodegenerative disorders and aging (Adamu et al., 2016).

The reverse phase HPLC-DAD analysis of the BG extract shows the presence of some phenolic compounds. A total of nine peaks appeared with retention times (Rt) of 3.32, 3.92, 5.53, 5.98, 6.49, 6.97, 7.77, 9.03 and 10.53 min, respectively (Figure 1). The most prominent peak Rt = 3.32 min corresponds to betulinic acid (Figure 2), a well-known anticancer agent which is derived from plant (Biswas et al., 2015). This is in

agreement with the report of Enwerem et al. (2001), who isolated betulinic acid from the leaf of *B. grandiflora*. Three other peaks corresponded with that of known antioxidant phenolic compounds, caffeic acid (Rt = 6.49 min), rutin (Rt = 6.97 min) and ferulic acid (Rt = 7.76 min) (Figures 3 and 4). Caffeic acid, rutin and ferulic acid have been reported to exhibit antioxidant activity (Rice-Evans et al., 1997).

Antioxidant activity

From Figure 5, 125 and 250 $\mu\text{g/mL}$ of the *B. grandiflora* extract exhibit antioxidant activity of 59 and 60%, respectively. While the control drug, ascorbic acid,

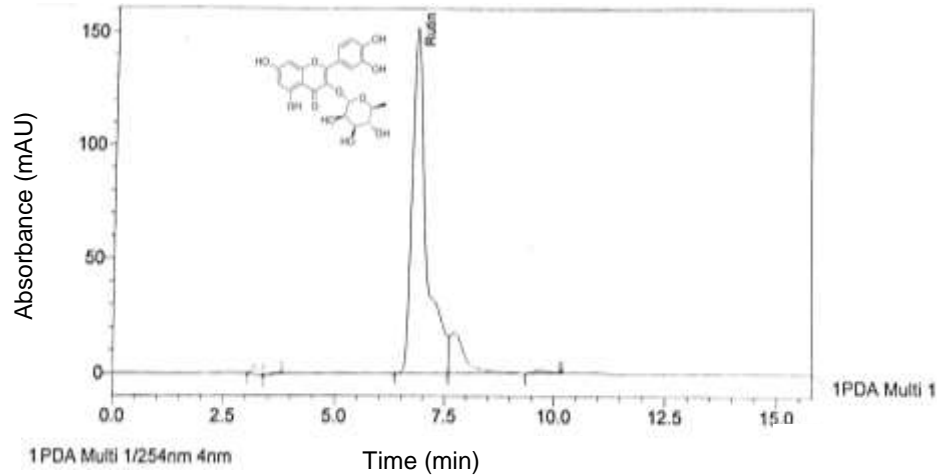


Figure 3. HPLC-DAD Chromatogram of Rutin.

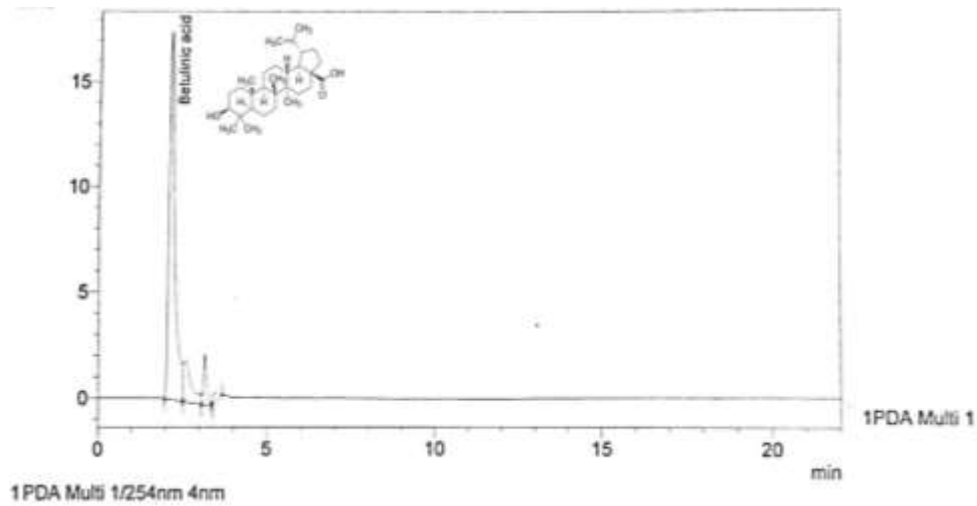


Figure 4. HPLC-DAD Chromatogram of Betulinic acid.

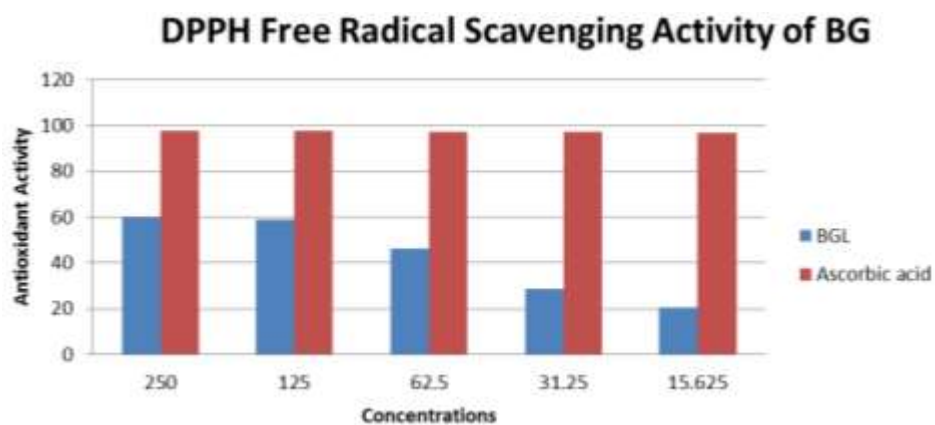


Figure 5. The DPPH free radical scavenging capacity of *B. grandiflora*. BGL: *B. grandiflora* leaves extract.

Table 1. The result of the IC₅₀ value of antioxidant assay of the leaf of *B. grandiflora*.

Extract	DPPH free radical scavenging (µg/mL)	Nitric oxide inhibition (µg/mL)	Reducing capacity (µg/mL)	Total phenolic content GAE (mg/g)
BGL	88.3	99	15	75.1
Ascorbic acid	0.7	3.4	7.4	-

Table 2. Percentage inhibition and percentage growth for *Sorghum bicolor* seeds treated with *B. grandiflora* extract.

Test solution	Time					
	48 h	72 h	96 h	48 h	72 h	96 h
	% Inhibition			% Growth		
0.5 mg/mL BG	44.5±2.6 ^e	45.5±2.0 ^e	66.9±2.3 ^e	55.5±2.6 ^b	54.5±2.0 ^b	33.1±2.3 ^b
1 mg/mL BG	64.3±2.3 ^d	54.0±2.0 ^d	70.6±2.4 ^d	35.7±2.3 ^c	46.0±2.0 ^c	29.4±2.4 ^c
2 mg/mL BG	88.0±1.0 ^c	83.2±2.1 ^c	85.8±1.5 ^c	12.0±1.0 ^d	16.8±2.1 ^d	14.2±1.5 ^d
4 mg/mL BG	93.1±0.6 ^a	95.6±0.7 ^a	98.6±0.7 ^a	6.9±0.6 ^f	4.4±0.7 ^f	1.4±0.7 ^f
Control (H ₂ O)	0.0 ^f	0.0 ^f	0.0 ^f	100 ^a	100 ^a	100 ^a
0.05 mg/mL Met	90.7±1.2 ^b	90.7±0.8 ^b	93.6±1.8 ^b	9.3±1.2 ^e	9.3±0.8 ^e	6.4±1.8 ^e

Values are means of triplicate determinations and are expressed as mean ± standard deviation; Means with different letters superscripts within each column differ significantly ($p < 0.001$) from one another. BG: *B. grandiflora*; Met: methotrexate.

exhibits an antioxidant activity of 97.6 and 97.8%, respectively.

In this assay, the ethanol extract of *B. grandiflora* leaves shows some degree of free radical scavenging capacity. At 250 and 125 µg/mL, the free radical scavenging capacity of the extract is above 50%. However, when compared with the reference compound ascorbic acid (IC₅₀ = 0.7 µg/mL), *B. grandiflora* (IC₅₀ 88.3 µg/mL) exhibited a weaker free radical scavenging capacity. The result of this study is similar to the report of Ode et al. (2013).

Nitric oxide is a potent inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, and inhibition of platelet aggregation and regulation of cell-mediated toxicity; it is also implicated in inflammation, cancer and other pathological conditions (Esiebo et al., 2018). From Table 1, *B. grandiflora* showed very weak nitric oxide (NO) inhibition (IC₅₀ 99 µg/mL) when compared with the NO inhibition of ascorbic acid (IC₅₀ = 3.4 µg/mL).

Several studies have confirmed the correlation between antioxidant activity and reducing power in plant extracts. Reducing power may be used as a pointer to potential antioxidants. The occurrence of antioxidants (reducing species) in extracts causes reduction of Fe (III) to Fe (II) complexes which can be determined by the formation of prussian blue color. This reduction may be due to the donation of a hydrogen atom from phenolic compounds (Shimada et al., 1992) which also correlates to the presence of a reducing agent. The reducing power of *B. grandiflora* (IC₅₀ = 15 µg/mL) is low compared to that of ascorbic acid (IC₅₀ = 7.4 µg/mL).

The total phenol content in BG extract expressed in

gallic acid equivalent (GAE) was 75.1 mg/g (Table 1). Plant phenols are a major group of the compounds acting as free radical scavengers and as such, it is important to estimate the content of phenol in the extract (Brum et al., 2013). Phenols contain hydroxyls that are responsible for the antioxidant properties which may be due to their redox properties (Rice-Evans et al., 1997). According to this study, the phenol content may be responsible for the antioxidant activity of the extract.

Growth inhibitory effects of *B. grandiflora* (leaves) on *S. bicolor* (Guinea corn) seeds

The result of antiproliferative activity of BG showed that there is a significant reduction in the length of radicles of *S. bicolor* seeds treated with the various concentrations of the extract when compared with the length of radicles of *S. bicolor* seeds treated with only distilled water. After 48 h of incubation, the percentage inhibition of 0.5, 1.0, 2.0 and 4.0 mg/mL of ethanol extract of BG was 44.5, 64.3, 88.0, and 93.1%, respectively. However, methotrexate has a percentage inhibition of 90.7% after 48 h of incubation. It was observed that the percentage inhibition increased with corresponding increase in the concentration of the extract (Table 2). This suggests that the extract exhibits a concentration-dependent inhibition. The extract also showed a time-dependent inhibition; the percentage inhibition of each concentration increases with increase in incubation time; the percentage inhibition after 48, 72 and 96 h for 0.5 mg/mL extract test solution was 44.5, 45.5 and 66.9%, respectively; that of 1 mg/mL was 64.3, 54 and 70.6%, respectively. 2 mg/mL exhibited

88.0, 83.2, and 85.8%, respectively and 4 mg/mL exhibited 93.1, 95.6 and 98.6%, respectively. Furthermore, the percentage inhibition for 2 and 4 mg/mL of the extract solution was similar to the percentage inhibition of the positive control (0.05 mg/mL methotrexate) which is a well known anticancer drug. The IC₅₀ values of the antiproliferative activity of the ethanol extract of BG were 0.50, 0.59 and 0.29 µg/mL after 48, 72 and 96 h of treatment, respectively. Therefore it can be implied that the ethanol extract of BG can mitigate the fast proliferation of cancer cells. The fact that 0.05 mg/mL of methotrexate showed a significant percentage inhibition justifies the use of this method.

The results of this study have shown that the ethanol extract of *B. grandiflora* contains phytochemicals which possess antioxidant properties and are reported to exert anticancer effects too. It also supports the report of the ethnomedicinal use of *B. grandiflora* for the management of tumors in South-west Nigeria (Soladoye et al., 2010). However, this is the first work reporting the antiproliferative activity of the plant. Furthermore, this is the first study to report the presence of some plant polyphenols specifically; rutin, ferulic acid and caffeic acid in *B. grandiflora*. Rutin is widely found in plants as glycoside, combining the flavonol quercetin and the disaccharide rutinose. The ability of flavonoids to act as potent antioxidants depends on their molecular structures, the position of the hydroxyl group and other features in their chemical structure (Iqbal et al., 2015). Ferulic acid and caffeic acid are also phenolic compounds. High content of phenolic compound has been linked to anticancer activities (Iqbal et al., 2015). Polyphenolic compounds have an aromatic benzene ring with substituted hydroxyl groups and other functional derivatives. These functional groups can bind to free radicals and also metal ions that could catalyze formation of reactive oxygen species (ROS) which promotes lipid peroxidation.

The antiproliferative activity of the plant could be due to the presence of betulinic acid which is the major constituent of *B. grandiflora* from HPLC analysis. Previously, it has been reported that betulinic acid from *Zizyphus* species namely; *Zizyphus rugosa*, *Zizyphus oenoplia* and *Zizyphus mauritian* showed excellent anticancer properties (Fulda, 2008). Furthermore, betulinic acid was reported to be a highly selective growth inhibitor of human melanoma, neuroectodermal and malignant tumor cells and to also induce apoptosis in these cells (Yogeeswari and Sriram, 2005). Betulinic acid has been identified as a potent anticancer agent and is currently undergoing clinical trials as an anticancer drug (Biswas et al., 2015).

Conclusion

The ethanol extracts of *B. grandiflora* leaves exhibited significant growth inhibitory effects on fast proliferating

cells (*S. bicolor* seed radicle). This suggests that it can inhibit cancerous cells. This study provided preliminary evidence that supports the ethnomedicinal use of BG in the treatment of tumor. Furthermore, the presence of betulinic acid, rutin, caffeic acid and ferulic acid may be working in synergy with all other phytochemicals to exert the antiproliferative activity of the plant. The authors intend to conduct subsequent studies on cancer cell line.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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