REPORT

Cytotoxic flavonoids from the young twigs and leaves of *Caesalpinia bonduc* (Linn) Roxb

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Abstract: The extraction, fractionation and recognition of flavonoids from the ethanolic extract of young twigs and leaves of *C. bonduc* were carried out. In addition, cytotoxic study of the flavonoids on two cancer cell lines, BGC-823 and HeLa was carried our using sulphorhodamine B assay. Seven flavonoids, six of which are being reported for the first time in this plant, were isolated. Their structures were identified by MS and NMR spectroscopic methods. Petroleum ether, ethyl acetate and water fractions exhibited moderate cytotoxic activity against HeLa cells. Five compounds showed cytotoxic activity against HeLa cell in comparison with Paclitaxel, while only one compound showed a good degree of cytotoxic activity against BGC-823 cell in comparison to Paclitaxel. The results obtained showed a structure - activity relationship.

Keywords: *Caesalpinia bonduc*; flavonoids; cytotoxic activity.

INTRODUCTION

The second leading cause of death in the United States of America is cancer; this is exceeded only by cardiovascular disease (Jemal *et al*., 2005). Since 1990, cancer incidence has resulted in a 22% increase in death rate in the four most frequent cancers namely, stomach, colorectal, lung and breast (Parkin *et al*., 2001). In the year 2000, more than nine million new cases of cancer were reported, resulting in, over six million death (Parkin *et al*., 2001). *Caesalpinia bonduc*, (family: Caesalpiniaceae, genus Fabaceae), commonly known as Gray Nicker nut (English) and Ayo (Yoruba, Nigeria), is a prickly shrub with a hard, grey, globular shaped and smooth shining surface seeds (Nadkarni, 1954). It is a medicinal plant predominantly dispersed in the tropical and subtropical parts of Africa, Asia and the Caribbean (Gupta *et al*., 2003). It has a lot of applications in folk medicine.

The pharmacological screening of the plant extracts reveals their anticancer, antioxidant, antimalarial, antihyperglycemic, anti-inflammatory, antirheumatic, antipyretic and anticonvulsant activities (Adesina, 1982; Gupta *et al*., 2004; Sonibare *et al*., 2009). Jäger and Saaby (2011), recently reported the anti-depressant, anti-anxiety, memory inducer and relaxing enhancer of *C. bonduc*. Previous investigations on the phytochemical constituents from *C. bonduc* has led to the isolation of various compounds including Bonducellin (Purushothaman *et al*., 1982), Caesalpinia-F (Pascoe *et al*., 1986), Bonducellipins-A to -D (Peter *et al*., 1997), Caesalpin-G and -H (Peter *et al*., 1998), Caesalpinolide-A to -E (Yadav *et al*., 2007; Yadav *et al*., 2009) and others. As a result of the reported anticancer activity of *C. bonduc* against Ehrlich ascites carcinoma (Gupta *et al*., 2004) and the dearth of information on its bioactive constituents from Nigeria, this research work was proposed and implemented. This article describes the in vitro cytotoxic activity of extracts and flavonoids isolated from young twigs and leaves of *C. bonduc* obtained from Nigeria against BGC-823 (Human gastric carcinoma) and HeLa (Human cervical adenocarcinoma) cell lines.

MATERIALS AND METHODS

Plant material

Young twigs and leaves of *C. bonduc* (Linn) Roxb. were collected from Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state, Nigeria. Plant identification was done by Dr. Conrad Omonhinmi, Department of Biological Sciences, College of Science and Technology,

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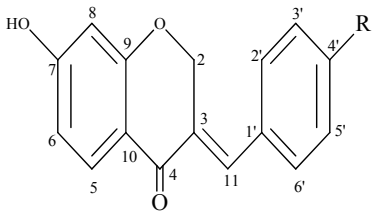
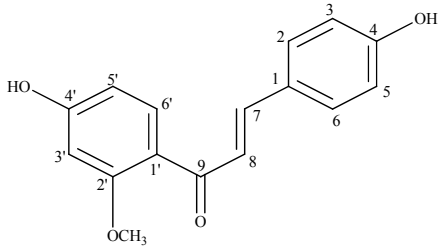
Covenant University, Ota, Ogun state, Nigeria. Authentication and voucher referencing were carried out at FRIN with voucher specimen SHI108408 deposited in their Herbarium.

Reagents, equipments and cell lines

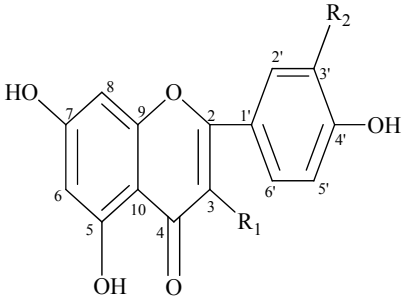
The reagents were of analytical grade. Paclitaxel, trichloroacetic acid (CCl_3COOH), glacial acetic acid and sulforhodamine B were bought from Sigma Chemicals (St Louis, MO, USA). Records of ^1H and ^{13}C NMR spectra were carried out on Bruker DRX-500 or 400 spectrometers (Bruker, Karlsruhe, Germany). Tetramethylsilane (TMS, $SiCH_3$) was used as internal standard; chemical shift (δ) and coupling constant (J) were measured in ppm and Hz respectively. Positive Electron Spray Ionization Mass Spectroscopy (ESI-MS) spectra were recorded on API Qstar time of flight pulsar instrument (Applied Biosystems, USA). Column chromatography (cc) was carried out over LH-20 Sephadex (Pharmacia Fine Chemical Co. Sweden) and silica gel (100 to 200, 200 to 300 mesh and 10 to 40µL, Qingdao Marine Chemical, Inc, China). Medium pressure liquid chromatography (MPLC) was carried out over Lichroprep Reverse phase gel RP - 18 (40-63 µm, Merck, Darmstadt, Germany). High-pressure liquid chromatography (HPLC) (HP Agilent 1100, Agilent Technologies, USA) was carried out over YMC-Pack ODS-A column. BGC-823 and HeLa cells were received from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (CAS), Shanghai, China. Cancer



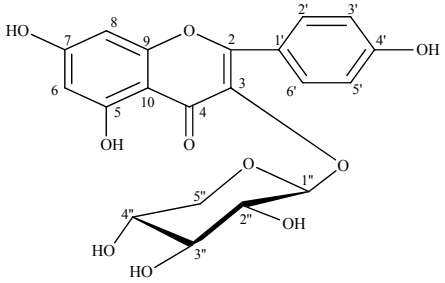
Compound 1, R = OCH_3
Compound 3, R = OH



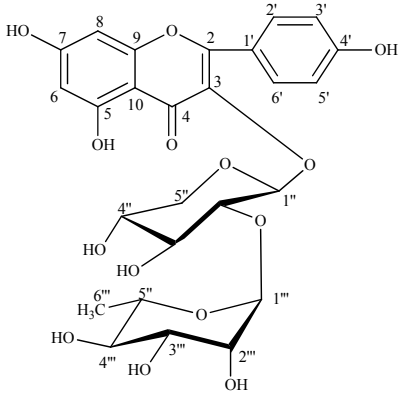
Compound 2



Compound 4, R1 = H, R2 = OH
Compound 5, R1 = OCH3, R2 = OH



Compound 6



Compound 7

Fig. 1: Chemical structures of compounds 1 – 7 isolated from *C. bonduc*

cell lines were cultivated in RPMI 1640 medium supplemented with 10% fetal blood saline (FBS) under a moisturised atmospheric state of 5% CO_2 at 37°C. Solvents were sterilised by filtering with 0.22 micron-pore Millipore filter in aseptic operation. Samples were prepared as stock solution by aseptic dissolution in dimethyl sulfoxide to concentration of 2.5mg/ml.

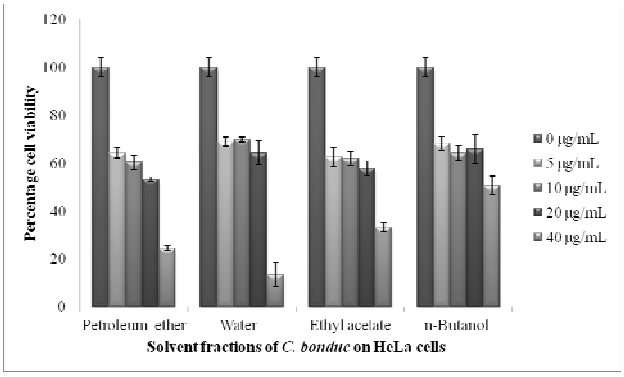


Fig. 2: The graph of percentage cell viability (Y axis) against concentrations of solvent fractions of *C. bonduc* (X axis) showing the cytotoxic effect of solvent fractions on HeLa cells. The values were presented as absorbance values ± standard deviation of triplicate experiments.

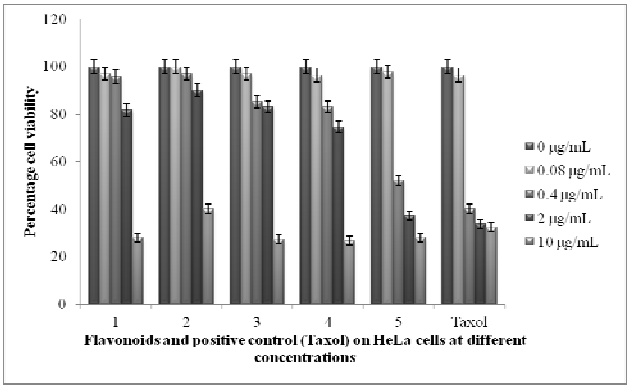


Fig. 3: The graph of percentage cell viability (Y axis) against concentrations of flavonoids (X axis) showing the cytotoxic effect of flavonoids on HeLa cells in comparison with Paclitaxel (Taxol). Compounds 6 and 7 have no inhibitory activity at the highest tested dose hence, they were not evaluated for further cytotoxic assessment. The values were presented as absorbance values ± standard deviation of triplicate experiments.

Extraction and fractional process of the plant

The young twigs and leaves of the plant collected were air dried at room temperature and powdered. Powdered plant (8.8kg) was extracted with 75% v/v ethanol (50L), at normal room temperature (25°C), by maceration for 72 hours using three consecutive extractions. The total filtrate was concentrated to dryness with rotary evaporator at 50°C. The dried ethanolic extract of the plant (1120g) was re-suspended in distilled water (H_2O) and partitioned in sequence on petroleum ether, ethyl acetate, and n-butanol. The different solvent fractions were concentrated with rotary evaporator to yield H_2O-soluble fraction (630g), petroleum ether-soluble fraction (150g), n-butanol-soluble fraction (170g) and ethyl acetate-soluble fraction (120g). Each fraction was subjected to cytotoxic activity determination.

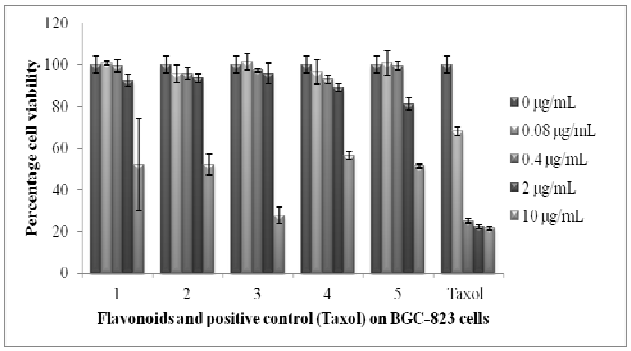


Fig. 4: The graph of percentage cell viability (Y axis) against concentrations of flavonoids (X axis) showing the cytotoxic effect of flavonoids on BGC-823 cells in comparison with Paclitaxel (Taxol). Compounds 6 and 7 have no inhibitory activity at the highest tested dose hence; they were not evaluated for further cytotoxic assessment. The values were presented as absorbance values ± standard deviation of triplicate experiments.

Purification and isolation of compounds

Experimental procedure of purification and isolation of compounds were described in the supplementary section of the Journal.

In vitro cytotoxic assay

The method of sulphorhodamine B (SRB) assay as described by Tang *et al*., 2010, was followed for a quantitative measurement of cell viability and maturation. Cultivated cancer cells in RPMI 1640 culture medium (Sigma), were seeded in aliquot part of 90µl in a 96-well microtiter plates at 4×10^4 cells/well. After twenty-four hours of culture, the microtiter plates were made up to 100µl with samples to the final concentrations of 10 µg/ml for pure compounds and 40µg/ml for solvent fractions. The mixture was incubated for two days. Fixation of cells were carried out by adding 25µl of 80% ice-cold trichloroacetic acid (CCl_3COOH, TCA) to each well, followed by incubation for 5 minutes and refrigeration at 4°C for one hour. Thereafter, the plates were rinsed, air-dried and stained with 100µl of Sulforhodamine B (SRB) (1% glacial acetic acid with 0.4% SRB) for 15 minutes. Washing to remove excess dye was carried through with the addition of 1% glacial acetic acids. Stained SRB cells were dissolved by the addition of Tris (100µl of 10mM) to each well. The absorbance of the plates carried out using a micro-plate spectrometer (Molecular Devices, SPECTRA MAX 340, USA) at 560 nm. Advance judgment was carried through using four different concentrations (dilution proportion 1:2) for samples with percentage inhibition (I%) of 50% and above, to calculate the IC_{50} rate (50% inhibitory

Table S1: ^{1}H and ^{13}C-NMR spectral data for compounds 1 and 3 (δ in ppm, J in Hz)

Compounds	δ_C	δ_H	δ_C	δ_H
	1		3	
2	67.53	5.35 (2H, d, J = 1.5)	69.05	5.35 (2H,br, s)
3	126.50	-	127.07	-
4	179.52	-	183.06	-
5	129.39	7.73 (1H, d, J = 8.5)	130.69	7.80(1H,d,J=8.0)
6	111.13	6.54 (1H, dd, J = 8.5, 2.0)	112.16	6.52 (1H, d, J = 8.0)
7	164.61	-	166.69	-
8	102.41	6.31 (1H, d, J = 2.0)	103.60	6.31 (1H, br, s)
9	162.47	-	164.85	-
10	114.30	-	115.90	-
11	135.21	7.63 (1H, br, s)	138.16	7.71 (1H, s)
1'	128.82	-	129.56	-
2'	132.19	7.39 (2H, d, J = 8.5)	133.50	7.25 (2H, d, J= 7.6)
3'	114.23	7.04 (2H, d, J = 8.5)	116.70	6.88 (2H, d, J= 7.6)
4'	160.26	-	160.53	-
5'	114.23	7.04 (2H, d, J = 8.5)	116.70	6.88 (2H,d, J=7.6)
6'	132.19	7.39 (2H, d, J = 8.5)	133.50	7.25 (2H,d, J=7.6)
4'-OCH_3	55.33	3.81 (3H, s)	-	-

^{1}H NMR and ^{13}C NMR were recorded at 500 and 125 MHz respectively in DMSO-d_6.

concentration). Cell growth suppression rates were showed as IC_{50}. A clinically used anticancer agent, Paclitaxel, was utilised as a positive control.

RESULTS

Fig. 1 illustrates the seven flavonoids (1 - 7) that were separated from the crude ethanolic extract of *C. bonduc.* Fig. 2 depicts bar chart illustrating the percentage viability of HeLa cells on solvent fractions of *C. bonduc*. Table 1 shows that cytotoxic activity (IC_{50}) of solvent fractions and compounds isolated from ethanolic extract of *C. bonduc*. Petroleum ether, water, compounds 1 - 5) and Taxol exhibited different levels of cytotoxic activities on HeLa cell lines with half maximum inhibitory concentration (IC_{50}) values ranging from 32 to 1.022 µg/mL while only compound 3 and Taxol exhibited cytotoxic activity on BGC 823 cell lines. Figs. 3 and 4 illustrate the effect of flavonoids isolated from *C. bonduc* on viability of HeLa and BGC-823 cells respectively.

DISCUSSION

Compound 1 was separated as a pale yellow crystalline solid with yield of 319.30 mg. Its chemical formula was ascertained to be $C_{17}H_{14}O_4$, on the ground of the molecular ion peak of positive ESI-MS m/z 305 $[M+Na]^+$. Compound 1 was elucidated as 7-hydroxy-4'-methoxyl-3,11-dehydrohomoisoflavanone (fig. 1). The 1D ^{1}H and ^{13}C NMR spectra data (table S1) were in accord with published work (Purushothaman *et al*., 1982). Compound 2 was separated as a pale yellow powder with yield of 15.60 mg. Its chemical formula was ascertained to be $C_{16}H_{14}O_4$, on the ground of the molecular ion peaks of positive ESI-MS m/z 271 $[M+H]^+$ and 293 $[M+Na]^+$. Compound 2 was identified as 4,4'-dihydroxy-2'-methoxy-chalcone (fig. 1). Its 1D ^{1}H and ^{13}C NMR spectra data (table S2) were in accord with published work (Namikoshi *et al*., 1987a). Compound 3 was separated as a pale yellow powder of 10.4mg yield. Its chemical formula was observed to be $C_{16}H_{12}O_4$, on the ground of the molecular ion peak of positive ESI-MS m/z 269 $[M+H]^+$. Compound 3 was identified as 7,4'-dihydroxy-3,11-dehydrohomoisoflavanone (fig. 1). The ^{1}H and ^{13}C NMR spectra data (table S1) were marked with close similarity with those of compound 1. The data were in agreement with published work (Namikoshi *et al*., 1987b).

Compound 4 was separated as a pale yellow powder like compounds 2 and 3 with yield of 12.80 mg. Its chemical formula was observed as $C_{15}H_{10}O_6$ from its molecular ion peak at positive ESI-MS m/z 287 $[M+H]^+$. Compound 4 was elucidated as Luteolin (fig. 1). The ^{1}H and ^{13}C NMR spectra data (table S3) were in accord with published work (Wagner and Chari, 1976). Compound 5 was separated as a pale yellow crystalline solid with yield of 227.00mg. Its chemical formula was observed to be $C_{16}H_{12}O_7$, on the ground of the chemical ion peak at positive ESIMS m/z 317 $[M+H]^+$. Compound 5 was elucidated as quercetin-3-methyl ether (fig. 1). The ^{1}H and ^{13}C NMR spectra data (table S3) were in accord with published work (Jurd and Horowitz, 1957).

Compound 6 was separated as a brown paste with yield of 24.40 mg. Its chemical formula was observed to be $C_{20}H_{18}O_{10}$, on the ground of the molecular ion peak at positive ESI-MS m/z 441 $[M+Na]^+$. Compound 6 was elucidated as kaempferol-3-O-β-D-xylopyranoside (fig. 1). The ^{1}H and ^{13}C NMR spectra data (table S4) were in

accord with published work (Kruglii and Glyzin, 1968). Compound 7 was separated as a pale yellow crystalline solid like compounds 1 and 5, with yield of 79.50 mg. Its chemical formula was observed to be $C_{26}H_{28}O_{14}$, on the ground of the molecular ion peak at positive ESIMS m/z 565 $[M+H]^+$. Compound 7 was identified to be Kaempferol-3-O-α-L-rhamnopyranosyl-1→2)-β-D-xylopyranoside (fig. 1). The ^1H and ^{13}C NMR spectra data were (table S4) in accord with published work (Moon *et al*., 2010).

Table S2: ^1H and ^{13}C-NMR spectral data for compound 2 (δ in ppm, J in Hz)

Compounds	δ_C	δ_H
	2	
1	128.04	-
2	131.38	7.50 (2H, d, J = 8.4)
3	116.87	6.82 (2H, d, J = 8.4)
4	161.21	-
5	116.87	6.82 (2H, d, J = 8.4)
6	131.38	7.50 (2H, d, J = 8.4)
7	144.16	7.56 (1H, d, J = 15.6)
8	125.07	7.41 (1H, d, J = 15.6)
9	193.16	-
1'	121.76	-
2'	162.53	-
3'	100.11	6.51 (1H, br s)
4'	164.49	-
5'	108.91	6.45 (1H, d, J = 8.4)
6'	133.73	7.57 (1H, d, J = 8.4)
2'-OCH_3	56.14	3.88 (3H, s)

^1H NMR and ^{13}C NMR were recorded at 500 and 125 MHz respectively in DMSO-d_6.

Table 1 indicates that compounds 1-5 showed good cytotoxic inhibition in their activities as indicated by their IC_{50} values while only compound 3 showed good cytotoxic inhibition against BGC-823 cells. On the other hand, compounds 6 and 7 showed insignificant inhibition (IC_{50} >10μg/mL) in both cell lines. The cytotoxicity result of compounds 4 and 5 are supported by the reports by Rubio *et al*., (2006) and Kawaii *et al*., (1999). However Rubio *et al*. (2006), reported that Kaempferol-3-methyl ether has cytotoxic activity with IC_{50} value of 35μM on HeLa cells while its glycones (compounds 6 and 7) lack cytotoxic activity. It is suggested that the lack of cytotoxic activity by compounds 6 and 7 might be due to the additional sugar moiety attached at position 3 of the C-ring thereby increasing their polarity and limiting their cellular permeability (Spencer, 2003). Increased molecular weight of compounds 6 and 7 might also limit their cellular permeability. From the comparison of cytotoxic activity of compounds 1 and 3 on BGC-823 cells, the reduced cytotoxic activity observed in compound 1 might be due to the additional methoxyl group at 4' position. Structure-activity relationship analysis of flavonols and flavones separated from *C. bonduc* suggests increased cytotoxic activity with the presentation of a hydroxyl group at 3' of the B ring and also with enhanced activity with methylation of the 3 hydroxyl group at C ring.

A number of bioactive flavonoids as well as other phytochemicals have been separated from *C. bonduc* with anticancer activity (Yadav *et al*., 2007; Yadav *et al*., 2009); nevertheless, this is the first report on the isolation, purification and identification of compounds 2 to 7 from *C. bonduc* in literature. In addition, this is also the first report on the cytotoxic activity of compound 3, 7,4'-

Table S3: ^1H and ^{13}C-NMR spectral data for compounds 4 and 5 (δ in ppm, J in Hz)

Compounds	δ_C	δ_H	δ_C	δ_H
	4		5	
2	166.00	-	158.0	-
3	103.82	6.54 (1H, s)	139.52	-
4	183.85	-	180.01	-
5	163.19	-	163.08	-
6	100.09	6.20 (1H, d, J = 1.2)	99.75	6.20 (1H, d, J = 2.0)
7	166.33	-	165.94	-
8	94.97	6.44 (1H, br s)	94.70	6.39 (1H, d, J = 2.0)
9	159.40	-	158.42	-
10	105.28	-	105.83	-
1'	123.64	-	122.88	-
2'	114.11	7.38 (1H, overlap)	116.42	7.63 (1H, d, J = 2.0)
3'	147.11	-	146.50	-
4'	150.98	-	149.97	-
5'	116.75	6.90 (1H, d, J = 8.4)	116.42	7.53 (1H, dd, J = 8.4)
6'	120.28	7.38 (1H, overlap)	122.31	6.90 (1H, d, J = 8.4)
3-OCH_3	-	-	60.52	3.78 (3H, s)

Table S4: ^{1}H and ^{13}C-NMR spectral data for compounds 6 and 7 (δ in ppm, J in Hz)

Compounds	δ_C	δ_H	δ_C	δ_H
	6		7	
2	158.38	-	158.12	-
3	135.31	-	134.30	-
4	179.36	-	179.12	-
5	163.00	-	162.90	-
6	99.89	6.18 (1H, br s)	99.82	6.06 (1H, br s)
7	165.96	-	165.50	-
8	94.76	6.37 (1H, br s)	94.71	6.24 (1H, br s)
9	15890	-	158.54	-
10	105.61	-	105.80	-
1'	122.58	-	122.90	-
2'	132.20	8.01 (2H, d, J = 8.4)	132.01	7.92 (2H, d, J = 8.,5)
3'	116.11	6.86 (2H, d, J = 8.4)	116.12	6.82 (2H, d, J = 8.5)
4'	161.60	-	161.23	-
5'	116.11	6.86 (2H, d, J = 8.4)	116.12	6.82 (2H, d, J = 8.5)
6'	132.20	8.01 (2H, d, J = 8.4)	132.01	5.53 (1H, d, J = 7.0)
1"	104.64	5.16 (1H, *d*, J = 6.8)	101.22	5.53 (1H, d, J = 7.0)
2"	75.33	3.74 (1H, d, J = 4.8)	79.32	-
3"	77.52	3.48 (1H, t, J = 5.8)	77.90	-
4"	70.98	3.41 (1H, d, J = 8.2)	72.22	-
5"	67.20	3.76 (2H,s, J = 4.6)	66.90	-
1"'	-	-	102.53	5.21 (1H, br s)
2"'	-	-	71.34	-
3"'	-	-	72.33	-
4"'	-	-	74.01	-
5"'	-	-	70.01	-
6"'	-	-	17.73	-

^{1}H NMR and ^{13}C NMR were recorded at 500 and 125 MHz respectively in DMSO-d_6.

dihydroxy-3,11-dehydrohomoisoflavanone on BGC-823 cells. Since flavonoids display a huge array of cellular activity, several mechanisms have been proposed for their cytotoxicity including inhibition of DNA topoisomerase I/II activity, increased generation of reactive species (Wang *et al*., 1999), decrease level of redox active proteins (Lu *et al*., 2006), DNA oxidation and fragmentation, regulation of heat-shock-protein expression, cell cycle arrest, modulation of survival/proliferation pathways and activation of proapoptotic cellular factors (Ramos, 2007).

Flavonoids' anti-cancer activity has been reportedly associated with induction of apoptosis, which is characterised by early mitochondrial dysfunction and endoplasmic reticulum-stress-induced signaling factors (Choi *et al*., 2011). From the reports of Huang *et al*. (2011), reduced cancer cell viability of an isoflavone, Osajin, isolated from medicinal plant was characterised by the release of cytochrome c into the cytosol, the gradual decline of mitochondrial transmembrane potential, down-regulation of glucose-regulated protein Bcl-2 and 78 kDa (anti-apoptotic factors), stimulation of the activities of caspases-3, -4, -8 and -9 and up-regulation of Bax and Fas lig and (FasL) (pro-apoptotic factors). The ability of quercetin to promotes apoptosis in cancer cell by down-modulating the expression of heat shock protein 90, which, in turn, induces the suppression of development and cell death in prostate cancer cells while exercising no quantitative effect on normal prostate epithelial cells has been demonstrated (Aalinkeel *et al*., 2008).

CONCLUSIONS

C. bonduc possesses cytotoxic activity; its petroleum ether and water fractions contain the major cytotoxic constituents. Compounds 1 to 5, all flavonoids, separated from *C. bonduc* were part of bioactive principles that mediate the cytotoxic activity of the plants.

SUPPORTING INFORMATION

Experimental procedure of purification and isolation of flavonoids and their 1D spectra data (tables S1-S4) are available as Supporting Information.

Table 1: Cytotoxic activity of solvent fractions and compounds of ethanolic extract of *C. bonduc*

Samples/ Compounds	IC_{50} (µg/mL) Hela cells lines	IC_{50} (µg/mL) BGC 823 cell lines
Pet. Ether	[a]32.00	NA
Water	[a]30.14	NA
75% Et	NA	NA
n-Butanol	NA	NA
Ethyl ac.	NA	NA
1	[#]5.88	NA
2	[#]8.69	NA
3	[#]5.91	[#]6.45
4	[#]5.27	NA
5	[##]0.81	NA
6	NA	NA
7	NA	NA
Taxol	[##]1.022	[##]0.15

IC_{50} is 50 % inhibitory concentration; Pet. ether, 75% Et, Ethyl ac. represent petroleum ether, 75% ethanol (crude) and ethyl acetate extracts respectively. [##, #] & [a] signify very strong, strong and weak cytotoxicity activities of samples while NA signify not applicable. The values were in triplicates.

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