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In vitro Cancer Cell Growth Inhibition and Antioxidant Activity of *Bombax ceiba* (Bombacaceae) Flower Extracts

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The flowers of *Bombax ceiba* were investigated for their chemical composition, antioxidant effects and antiproliferative activity against seven human cancer cell lines. The antiproliferative responses of diethyl ether (DE) and light petroleum (PE) extracts were evaluated by sulforhodamine B (SRB) assay against MCF-7, HeLa, COR-L23, C32, A375, ACHN, and LNCaP cells in comparison with a human normal cell line, 142BR. Moreover, extracts were characterized by GC-MS analysis and tested for their antioxidant properties by different *in vitro* systems, namely DPPH, Fe-chelating activity and β-carotene bleaching test. Both PE and DE extracts showed the highest antiproliferative activity against human renal adenocarcinoma (ACHN) in a concentration-dependent manner. PE extract showed the highest radical scavenging activity against the DPPH radical, while DE extract was more active in the β-carotene bleaching test. The presence of β-sitosterol and some fatty acids may contribute to the bioactivity of *B. ceiba* flower extracts.

Keywords: *Bombax ceiba*, Flowers, GC-MS, Antiproliferative activity, Antioxidant activity.

In recent years, a significant part of drug discovery has been focused on compounds as potential agents for prevention and/or treatment of cancer. This is not surprising because, both in most developed and developing countries, cancer is amongst the three most common causes of death and morbidity. Natural products play a relevant role in cancer therapy with a significant number of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms [1]. In spite of important results, the search for improved antitumor agents continues to be an important line in the discovery of modern anticancer drugs and the evaluation of cytotoxicity represents a good starting point [2,3].

The interest in free radicals and their role in the tumor microenvironment has recently increased. Cancer and several inflammatory processes that lead to cancer and autoimmune diseases have been related to the direct and/or indirect effects of oxidative stress induced by free radicals. In normal conditions, reactive oxygen species (ROS) have an important role in normal cells in signal transduction and gene transcription. Nevertheless, ROS may act as a trigger for carcinogenesis. There are several anti-cancer drug candidates with antioxidant capacity [4]. Genistein, curcumin, and resveratrol are interesting examples.

Bombax ceiba L. (Bombacaceae), popularly known as the red silk cotton tree, is a medicinal plant belonging to the Bombacaceae family. This species has been used in traditional medicine for its emetic, diuretic, and demulcent properties [5,6]. Several studies reported the hepatic protective, antibacterial, antiviral, antioxidant, hypotensive, antiangiogenic, anti-hepatitis B virus (HBV), and anti-inflammatory effects [7-10]. A recent study demonstrated the potential role of the fruit of *B. ceiba* in the treatment of urolithiasis [11]. Phenols, sesquiterpenoids, steroids, naphthoquinones and neolignans have been isolated from different parts of *B. ceiba* [12,13]. The reported interest in the bioactive constituents obtained from the different parts of *B. ceiba* encouraged the investigation of its flowers. So, in this study, the flowers of *B. ceiba*, collected in Giza (Egypt), were investigated for the first time for their potential antiproliferative, radical scavenging, and metal-chelating activities.

The flowers of *B. ceiba* were extracted with light petroleum (PE) and diethyl ether (DE) with yields of 1.4 and 2.0%, respectively. Both extracts were analysed by gas chromatography-mass spectrometry (GC-MS). The DE extract was characterized by the presence of β-sitosterol (14.1%) as its main compound, followed by several alkanes and fatty acids namely palmitic acid, stearic acid, ethyl palmitate and ethyl linoleate (Table 1). Four monoterpenes, namely α-pinene, camphene, limonene and 1,8-cineole were also identified. β-Sitosterol (9.8%), palmitic acid (9.7%), stearic acid (8.8%), stigmasterol (6.6%), ethyl palmitate (4.1%) and ethyl linoleate (3.2%) were the main constituents identified in the PE extract. Myristic acid was identified only in this extract.

Both extracts were evaluated for their potential antiproliferative activity by using SRB assay against a panel of human cancer cell lines: human renal cell adenocarcinoma (ACHN), human Caucasian lung large cell carcinoma (COR-L23), human Caucasian lung carcinoma (A549), human Caucasian colon adenocarcinoma (Caco-2), human hepatocellular carcinoma (Huh-7D12), and amelanotic melanoma (C32). Data are reported in Table 2. *B. ceiba* non-polar extracts were able to inhibit the viability of tumor cell lines in a concentration-dependent manner (Figure 1). The more responsive cell line was the human renal adenocarcinoma cell line (ACHN) with IC_{50} values of 45.5 and 53.2 μg/mL for PE and DE, respectively. Interesting results were obtained also with the PE extract against the COR-L23 cell line with an IC_{50} value of 52.7 μg/mL, that is 1.1-fold higher than the positive control, vinblastine. A moderate activity was observed against LNCaP and HeLa cell lines. The less responsive cell line was MCF-7 with IC_{50} values of 62.8 and 127.3 μg/mL for DE and PE, respectively. None of the tested extracts affected the proliferation of the normal cell line, at the maximum concentration tested, suggesting a selective activity against the tumor cell lines.

Table 1: The main identified compounds in *B.ceiba* non-polar extracts.

Compound	DE[a])	PE[a])	Identification[b])
α-Pinene	0.7	0.5	A
Camphene	0.2	tr	A
Limonene	0.4	0.5	A
1,8-Cineole	0.3	tr	A
Dodecane	tr	0.3	A
1-Hexadecene	0.4	0.9	C
1-Octadecene	tr	2.3	C
6,10,14-Trimethyl-2-pentadecanone	0.5	0.2	C
Myristic acid	-	3.7	A
Palmitic acid	1.6	9.7	A
Ethyl palmitate	2.6	4.1	B
1-Octadecanol	0.5	-	B
Stearic acid	1.9	8.8	A
Ethyl linoleate	3.5	3.2	B
Tricosane	1.2	0.9	A
Tetracosane	0.4	1.6	A
Cyclotetracosane	0.4	0.6	B
Pentacosane	10.3	4.2	A
Hexacosane	1.5	-	A
Heptacosane	12.4	4.1	A
Octacosane	1.4	0.7	A
Nonacosane	11.7	5.4	A
Hexatriacontane	1.5	-	B
β-Sitosterol	14.1	9.8	A
Stigmasterol	4.2	6.6	A

[a]) Abundance calculated as % peak area mean values, mean ± standard deviation (n= 3). [b]) The constituent identification is indicated by the following: A, mass spectrum and retention time agreed with standard; B, mass spectrum and retention time agreed with database or literature; C, mass spectrum agreed with mass spectral database. tr: trace (< 0.1%).

Among the identified compounds in both extracts sterols and fatty acids represent the most abundant constituents. In recent years, a great deal of interest has been given to the role of phytosterols in the protection from cancer. β-Sitosterol demonstrated protection from chemically induced colon cancer and showed an inhibitory effect on tumor growth of HT-29 cells, a human colon cancer cell line [14,15]. Moreover, the effect of β-sitosterol and campesterol, compared with cholesterol, on the growth and apoptosis of MDA-MB-231, a human breast cancer cell line, has been investigated [16].

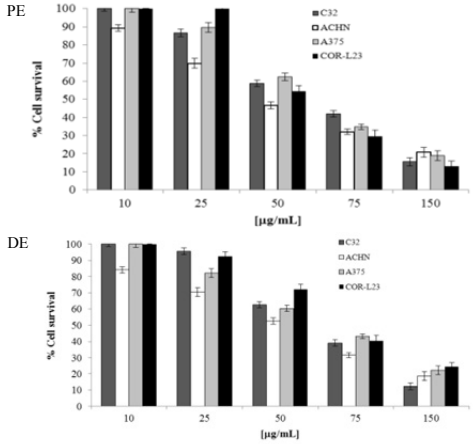

Figure 1: Antiproliferative activity of *B. ceiba* extracts against C32, ACHN, A375, and COR-L23 cell lines. DE: diethyl ether extract; PE: light petroleum extract. Data are mean ± SD (n= 3).

At concentrations in the range of 12.5-50.0 µg/mL, palmitic acid, isolated from a marine red alga, demonstrated cytotoxic activity against human leukemic cells. Additionally, palmitic acid induced apoptosis in the human leukemic cell line MOLT-4 and showed antitumor activity in mice. One molecular target of the fatty acid in tumor cells is DNA topoisomerase I [17].

A fraction composed of three free fatty acids, palmitic acid, (*Z*)-9-octadecenoic acid and octadecenoic acid, induced caspase-3 activation during apoptosis in colon 26 tumor cells [18].

Table 2: Antiproliferative activity (IC_{50} µg/mL) of *B. ceiba* non-polar extracts against a panel of human tumor cell lines.

Cell line	Extract		Positive control	
	DE	PE	Vinblastine	Taxol
MCF7	62.8 ± 2.8[a]	127.3 ± 4.1[a]	-	0.08 ± 0.006
ACHN	53.2 ± 1.4[a]	45.5 ± 3.0[a]	22.7 ± 0.5	-
C32	61.1 ± 2.1[a]	61.0 ± 2.2[a]	3.0 ± 0.1	-
A375	64.2 ± 1.3[a]	60.4 ± 2.7[a]	8.9 ± 0.1	-
LNCaP	66.4 ± 2.2[a]	70.3 ± 2.8[a]	29.3 ± 0.9	-
HeLa	76.6 ± 3.2[a]	61.3 ± 2.4[a]	-	15.5 ± 1.9
COR-L23	67.1 ± 3.2[a]	52.7 ± 3.5[a]	45.4 ± 2.3	-
142BR	> 100	> 100	> 100	> 100

Data are expressed as mean ± SD (standard deviation), (n= 3). Vinblastine and taxol were used as positive controls. MCF-7: One-way ANOVA ***p< 0.0001 (F= 1648 R^2= 0.998) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* taxol). ACHN: One-way ANOVA ***p< 0.0001 (F= 96.16, R^2= 0.969) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* vinblastine). C32: One-way ANOVA *** p< 0.0001 (F= 687.7, R^2= 0.996) Dunnett's Multiple Comparison Test [a]p< 0.01(DE, PE *vs* vinblastine). A375: One-way ANOVA *** p< 0.0001 (F= 759.9, R^2= 0.996) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* vinblastine). LNCaP: One-way ANOVA ***p< 0.0001 (F= 407.9, R^2= 0.993) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* vinblastine). HeLa: One-way ANOVA ***p< 0.0001 (F= 805.1, R^2= 0.996) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* taxol). COR-L23: One-way ANOVA ***p< 0.0001 (F= 85.0, R^2= 0.966) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE, *vs* vinblastine).

More recently, Takeara *et al*. [19] isolated from *Didemnum psammatodes* three methyl esters, four steroids, and two fatty acids, together with other constituents. The cytotoxic activity of these compounds was evaluated against acute promyeloblastic leukemia (HL-60), chronic myelogenic leukemia (K-562), lymphoblastic leukemia (CEM), and T-cell leukemia (Molt-4). The mixture of the three methyl esters was the most active showing IC_{50} values that ranged from 2.43 µg/mL to 9.96 µg/mL against Molt-4 and K-562 cell lines, respectively. Among the minor constituents identified in the non-polar extracts of *B. ceiba* were limonene and α-pinene. Limonene was tested against A549 and HepG2 cell lines demonstrating IC_{50} values of 0.59 and 0.89 mM, respectively [20]. α-Pinene recently showed antioxidant and anticancer activities [21,22]. The antiproliferative activity of our extracts may be related to the synergistic or cumulative effects of their components.

The *B. ceiba* extracts were analysed for their potential antioxidant effects by using three different *in vitro* assays. Both extracts showed DPPH radical scavenging activity in a concentration-dependent manner. PE extract was more active than DE extract with an IC_{50} value of 37.6 µg/mL (Table 3).

The extracts were also able to inhibit the discoloration of β-carotene in a concentration-dependent manner (Figure 2). In this assay, DE extract demonstrated the highest activity (IC_{50} of 58.3 µg/mL after 30 minutes of incubation). The IC_{50} of 93.8 and 90.7 µg/mL of PE indicated that its activity was not correlated with the time of heating. The Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex. The most active extract was DE with an IC_{50} value of 33.5 µg/mL. Our results may be partially explained by the presence of fatty acids as main constituents. A literature survey revealed the antioxidant properties of fatty acids [23,24].

The antioxidant activity of another *Bombax* species was previously studied. The flowers of *B. malabaricum* were extracted with water, 50% ethanol, and 80% acetone and investigated for their DPPH radical scavenging activity, oxygen radical absorbance capacity (ORAC), reducing power, and inhibition on phosphatidylcholine liposome peroxidation [25]. All the extracts possessed antioxidant capacity. The *n*-hexane and methanol extracts of *B. malabaricum* flowers were shown to scavenge the free radical DPPH with a percentage of 49.4% at a concentration of 0.55 and 85.3% at a concentration of 0.5 mg/mL, respectively [26].

Table 3: Radical scavenging, antioxidant and metal chelating activity (IC_{50}, μg/mL) of *B. ceiba* extracts.

B. ceiba	DPPH test	β-Carotene bleaching test		Fe^{2+} chelating activity test
		30 min	60 min	
DE	58.6 ± 2.6[a]	58.3 ± 3.6[a]	85.5 ± 2.9[a]	148.8 ± 4.9[a]
PE	37.6 ± 1.9[a]	93.8 ± 3.8[a]	90.7 ± 2.6[a]	33.5 ± 3.5[a]
Positive control				
Ascorbic acid	5.0 ± 0.8	-	-	-
Propyl gallate	-	1.0 ± 0.04	1.0 ± 0.04	-
EDTA	-	-	-	1.3 ± 0.05

Data are expressed as mean ± SD (standard deviation) (n= 3). Ascorbic acid, propyl gallate and EDTA were used as positive controls. DPPH test: One-way ANOVA ***p< 0.0001 (F= 1012 R^2= 0.997) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* ascorbic acid). β-Carotene bleaching test 30 min incubation: One-way ANOVA ***p< 0.0001 (F= 1710, R^2= 0.998) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* propyl gallate). β-Carotene bleaching test 60 min incubation: One-way ANOVA *** p< 0.0001 (F= 989.6, R^2= 0.997) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* propyl gallate). Fe^{2+}-chelating: One-way ANOVA *** p< 0.0001 (F= 3675, R^2= 0.999) Dunnett's Multiple Comparison Test [a]p< 0.01 (DE, PE *vs* EDTA).

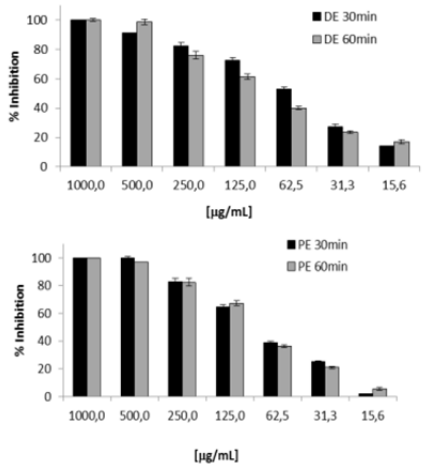

Figure 2: *B. ceiba* antioxidant activity evaluated by β-carotene bleaching test after 30 and 60 minutes of incubation. DE: diethyl ether extract, PE: light petroleum extract. Data are mean ± SD (n= 3).

The identification of natural compounds and the development of their derivatives have significantly contributed to the progress in the management of cancer. Natural compounds comprise classical cytotoxic moieties targeting nonspecific macromolecules expressed by cancer cells, compounds targeting macromolecules specifically expressed on cancer cells, and chemopreventive agents that may inhibit specific processes involved in carcinogenesis.

The search for improved cytotoxic agents continues to be an important line in the discovery of anticancer drugs. In this context we have analyzed the flowers of *B. ceiba* for their potential antiproliferative and antioxidant effects in relation to their chemical constituents. *B. ceiba* exhibited potential inhibition of human tumor cell growth and antioxidant capacity. These results encourage further studies in order to exploit this plant for pharmaceutical applications.

Experimental

Plant materials: The flowers of *B. ceiba* were collected in Giza (Egypt) in April 2012, and authenticated by Dr M. El-Gebaly, Department of Botany, National Research Centre (NRC), and Mrs T. Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of Orman Botanical Garden, Giza. A voucher specimen (143612) was deposited at the herbarium of NRC.

Extraction procedure: The flowers of *B. ceiba* (250 g) were dried, pulverized and exhaustively extracted in a Soxhlet apparatus using light petroleum and diethyl ether as solvents to obtain light petroleum (PE) and diethyl ether extracts (DE).

Gas chromatography-mass spectrometry (GC-MS) analysis: GC-MS analyses were carried out using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5 non polar capillary column (30 m × 0.25 mm × 0.25 μm) and interfaced with a Hewlett Packard 5973 Mass (EI, 70 eV) spectrometer using the following experimental conditions: oven temperature for 5 min at 50°C, then 50-250°C at a rate of 5°C/min; then held isothermal for 10 min. The injector and detector temperatures were 250 and 280°C, respectively. Helium was used as carrier gas. Constituents were tentatively identified by comparison of their mass spectra with those stored in the Wiley 138 library, with those of the literature, and with those of reference standard compounds.

Culture conditions and SRB assay: The protein-staining sulforhodamine B (SRB) assay was used for the measurement of cell proliferation, as previously described [27]. Human Caucasian lung large cell carcinoma COR-L23 (ECACC No.: 92031919), amelanotic melanoma C32 (ATCC No.: CRL-1585), malignant melanoma A375 (ECACC No. 88113005), renal cell adenocarcinoma ACHN (ATCC No. CRL-1611), hormone dependent prostate carcinoma LNCaP (ATCC No: CRL-1740), human breast cancer cell line MCF-7 (ATCC No.: HTB-22D), cervical cancer cell line HeLa (ATCC No.: CCL-2), and skin fibroblasts 142BR (ECACC No. 90011806) were used.

COR-L23, C32, ACHN, and LNCaP cells were cultured in RPMI 1640 medium, while MCF-7, HeLa, A375 and 142BR cells were cultured in D-MEM medium. Both media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. The cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. The optimal plating density of each cell line was determined over a concentration range of 2×10^4 to 5×10^4 to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance and cell number when analysed by the SRB assay.

DPPH assay: Radical scavenging capacity was determined according to the technique reported by Blois [28]. Ascorbic acid was used as positive control. The radical scavenging activity was calculated according to the equation: Scavenging activity= $[(A_0-A_1/A_0)\times 100]$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the extract.

β-Carotene bleaching test: Antioxidant activity was determined using the β-carotene bleaching test with some modifications [29]. Propyl gallate was used as positive control. The bleaching of β-carotene was measured and expressed as antioxidant activity (AA): $AA= [1-(A_0-At)/(A°_0-A°_t)]\times 100$, where A_0 and $A°_0$ are the absorbance values measured at the incubation t= 0 min for samples and control, respectively; A_t and $A°_t$ are the absorbance values of samples and control at t= 30 and 60 min, respectively.

Fe^{2+}-chelating activity assay: The chelating activity of *B. caiba* extracts for ferrous ions (Fe^{2+}) was measured according to a previously described method [30]. The chelating activity of extracts was calculated using the equation: Chelating rate = $(A_0-A_1)/A_0 \times 100$, where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract. EDTA was used as positive control.

Statistical analysis: The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression using GraphPad Prism version 4.0 for Windows (San Diego, CA, USA). The concentration-response curve was obtained by plotting the percentage inhibition *versus* concentration. Differences concerning parameters were analysed by the one-way ANOVA test to compare group means. To complete the statistical analysis, a multicomparison Dunnet's test was performed.

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