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LC-ESI-MS Analysis, Antitumor and Antiviral Activities of *Bosica senegalensis* Aqueous Methanolic Extract

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TWENTY ONE compounds (twelve flavonoids, four benzoylglucarate isomers, two phenolic acids, two glucosinolates, and one saccharide) were identified from the *B. senegalensis* aqueous methanolic extract using LC-ESI-MS technique. Among the identified compounds, quercetin-*O*-sinapoylglucoside-*O*-glucoside (**13**) and quercetin-di-*O*-benzoyl glucoside (**16**) are newly identified natural products.

B. senegalensis aqueous methanolic extract showed strong inhibitory effect against H5N1 viruses and moderate antitumor activity against three carcinoma cell lines.

Keywords: *Boscia senegalensis*, Capparaceae, LC-ESI-MS, Antitumor, Antiviral.

Introduction

The genus *Boscia* Lam. belongs to family Capparaceae comprises of approximately 37 species mainly distributed in tropical and Southern Africa except (*B. arabica Pest*.) distributed in southern Arabia [1, 2].

Boscia senegalensis (Pers.) Lam. ex Poir**.** is an evergreen shrub reaching 7m in height and distributed throughout Gabel Elba, Egypt; North-central and northern Senegal [3], the importance of the plant for the rural agro-economy in Africa for both human and animals [4] where it is traditionally used by farmers against stored grain insects. The biological activity of the plant is due to the liberation of MITC (methlyisothiocyanate) from a glucosinolate precursor glucocapparin contained in *Boscia* fruits and leaves. The introduction of the released MITC from *B. senegalensis* in the storage systems decreases the density of the population of parasites and increases the seed losses by allowing the development of the bruchid population [5].

Although the fruit of *B. senegalensis* is highly acidic; it is used for human consumption and becomes edible after soaking in water for seven days [6]. In Sudan, it is fermented into beer, while the leaves are used in food preservation against parasites [6].

Dried bark and leaves are used for schistosomiasis, while the leaves extract is used as eyewash seeds have antidiabetic properties mainly due to its high content of glucocapparin [6].

Phytochemical studies on *B. senegalensis* conducted on the leaves and fruits identified glucosinolate (methyl, 2-propyl, and 2-butyl-glucosinolate) [7], flavonol glycosides, megastigmane, monoterpenes, phenolic compounds and lignan [8].

The aim of the current study is to profile the phytochemical constituents of *B. Senegalensis* aqueous methanolic extract using LC-ESI-MS; in addition to investigating its antitumor and antiviral activities.

Experimental

Plant material

The leaves of *B. senegalensis* were collected from Gebel Elba, south of Egypt on March 2016. The authentication of plant sample was achieved by Prof. Dr. Salwa Kawashty, Phytochemistry and Plant Systematics Department. A voucher specimen (s.n SK 860), was deposited in the herbarium of National Research Center (CAIRO).

Human tumor cell lines

Human tumor cell line; Breast (MCF7), liver (HEPG2) and colon (HCT) were obtained from

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the American Type Culture Collection (ATCC, Minnesota, USA). The tumor cell lines were maintained at the National Cancer Institute, Cairo, Egypt by serial sub-cultures.

Preparation of extracts for bioassay

Stock solutions of the tested extract were dissolved as 0.1 g in 1 ml of 10% Dimethyl Sulfoxide (DMSO) in deionized water. The prepared extract solution was used for both cytotoxicity and antiviral bioassays.

MTT cytotoxicity and determinate assay (TC_{50})

Samples were 10-fold serially diluted with Dulbecco's Modified Eagle's Medium (DMEM). Stock solutions of the test extract were prepared in 10 % DMSO in ddH_2O. The cytotoxic activity of the extract was tested in Madin Darby Canine kidney (MDCK) cells by using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method [9] with minor modification. Briefly, the cells were seeded in 96 well-plates (100 µl/well at a density of 3×10^5 cells/ml) and incubated for 24 hr at 37 ºC in 5% CO_2. After 24 hr, cells were treated with various concentrations of the tested compounds in triplicates. After further 24 hr, the supernatant was discarded and cell monolayers were washed with sterile phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCl in 50 ml isopropanol). The absorbance of formazan solutions was measured at λ_{max} 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined as follows: % of cytotoxicity = [(Absorbance of cell without treatment - Absorbance of cell with treatment)/ Absorbance of cell without treatment] × 100. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC_{50}).

Plaque reduction assay

Anti-H5N1 activity of the extract of *B. senegalensis* was investigated by plaque reduction assay with confluent 24 h old monolayer of MDCK cells. Assay was carried out according to the reported methods [10]. In a six well plate where MDCK cells (10^5 cells / ml) were cultivated for 24 hrs at 37°C. A/Chicken/Egypt/M7217B/2013 (H5N1) virus was diluted to give 104 PFU/well and mixed with the safe concentrations of the tested compounds and incubated for 1 hour at 37°C before being added to the cells. Growth medium was removed from the cell culture plates and virus-Cpd or virus-extract and Virus-Zanamivir mixtures were inoculated (100 µl / well). After 1 hour contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose was added into the cell monolayer, plates were left to solidify and incubated at 37°C till formation of viral plaques (3 to 4 days). Formalin (10%) was added for two hours then plates were stained with 0.1% crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells and finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as follows: % of inhibition= [viral count (untreated) - viral count (treated)/viral count (untreated)] ×100.

Assay of the antitumor activity

Principle

The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara [11]. SRB is a right pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

Procedure

Cells were seeded in 96-well microtiter plates at initial concentration of 3×10^3 cell/well in a 150 µl fresh medium and left for 24 hours to attach to the plates. Different concentrations 0, 5, 12.5, 25, 50 µg/ml of drug were added. For each drug concentration, 3 wells were used. The plates were incubated for 48 hours. The cells were fixed with 50µl cold trichloroacetic acid 10% final concentration for 1 hour at 4 °C. The plates were washed with distilled water using (automatic washer Tecan, Germany) and stained with 50 µl 0.4 % SRB dissolved in 1 % acetic acid for 30 minutes at room temperature. The plates were washed with 1 % acetic acid and air-dried. The dye was solubilized with 100 µl/well of 10 M tris base (pH 10.5) and optical density (O.D.) of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader (Sunrise Tecan reader, Germany). The mean background absorbances was automatically subtracted and mean values of each drug concentration was calculated. The percentage of cell survival was calculated as follows: Surviving fraction = O.D.

(treated cells)/ O.D. (control cells). The IC_{50} values (the concentrations of drug required to produce 50 % inhibition of cell growth) were also calculated.

Extraction Process

The fresh leaves of *B. senegalensis* (1.15 kg) were air dried in shad and ground, then extracted three times at room temperature with 70% methanol/water. The aqueous methanol extract was evaporated under reduced pressure and temperature to obtain a residue of 204 g.

Acid hydrolysis

Acid hydrolysis was carried out for 60 minutes at 100 °C using 2N hydrochloric acid. The yielded aglycones were then extracted with ethyl acetate and identified by co-chromatography with stander samples; while the sugars obtained by hydrolysis of the flavonoid glycosides were identified by PC using BBPW (Benzene: n-butane: pyridine: water; 1:5:3:3), with a standard sugar mixture. The dried chromatograms were visualized by aniline phthalate reagent. The sugar spots were observed in daylight. The R_f values of tested sugars were compared with those of reference sugars.

LC-ESI-MS Analysis

LC-ESI-MS analysis: HPLC (Waters Alliance 2695) and MS spectrometry (Waters 3100). The defatted 70% methanol extract of *B. sensgalensis* solution (5 mg/ml) was prepared in HPLC grade solvent mixture of $CH_3CN/MeOH/H_2O$ (1:1:2; *v*/*v*/*v*) and filtered using membrane disc filter (0.45µm) and prepared for qualitative analysis. The mobile phase was prepared daily by filtering through 0.45 m membrane disc filter and degassed by sonication before use. The mobile phase for gradient elution consists of two solvents: solvent A (0.1% formic acid (FA) in H_2O) and solvent B (0.1% FA in CH_3CN/MeOH (1: 1; *v*/*v*)). The linear gradient profile was as follows: 95% A (5min), 95-90% A (10 min), 90-50% A (55 min), 50-95% A (65 min), and 95% A (70 min). The injection volume was 10 L. The flow rate (0.6 ml/min) was split 1:1 before the MS interface. The negative ion mode parameters were as follows: source temperature 150 3°C, desolvation temperature 350°C, cone gas flow 50 L/h, cone voltage 50 eV, capillary voltage 3 kV, and desolvation gas flow 600 L/h. Spectra were recorded in the ESI negative mode between 170/ 50-1000. The peaks and spectra were processed using the Maslynx 4.1 software. Unknown peak was tentatively identified by comparing its retention time and mass spectrum with literatures. Known peak was identified by comparing its retention time (t_R) and mass spectrum with a known standard**.**

Results and Discussion

Acid hydrolysis

The co-chromatography of the ethyl acetate extract gave three yellow spots under UV, indicating the flavonol nucleolus, and has the same R_f of rhamnocetrin, quercetin and rhamnetin aglycones. Glucose and rhamnose were detected as sugar moieties in the aqueous extract. The complete acid hydrolysis indicated that all glycosides were in *O*-glycoside form.

LC-ESI-MS analysis

Plant extracts generally occur as a combination of different types of phytochemicals or biologically active compounds with different polarities, their isolation and identification remain a big challenge. Liquid chromatography mass spectrometry (LC-MS) is a powerful and new technique for identification of the complex botanical extracts [12]. It provides information for structural elucidation of the components of these extracts. Therefore, in the present work, the defatted 70% methanolic extract of *B. senegalensis* was subjected to HPLC coupled with MS spectrometry in negative ion mode (Fig. **1**). The identification of the components of the extract was carried out through their retention times, molecular weights (MW), calculated *m/z* and major fragments which produced under the ionization conditions, as well as by comparison of these data with the previously reported data in the literatures.

Twenty one compounds were identified from the *B. senegalensis* aqueous methanolic extract (Table1, Fig. 1) using LC-ESI-MS technique. Peaks **1** and **7** which appeared with molecular ion peak at *m/z* 341, 325 respectively, are phenolic acid glycosides, after the loss of hexose moiety and the presence of fragments at *m/z* 179, 163 indicated that compound **1** is identified as caffeic acid glucoside while **7** is coumaric acid glucoside [13, 14]. Peaks **2** and **3** have the same molecular ion peak at *m/z* 412 and identified as glucocapparine sulfate isomers which confirmed by their fragmentation patterns in the literature [15]. Four benzoylglucarate isomers (**4-6, 8**) were identified by their pseudomolecular ions at *m/z* 313 $[M-H]^-$ and its product ions at *m/z* 191 [M-H-benzoic acid]$^-$, 147, 85 [16]. Peak **9** with molecular ion peak at *m/z* 371 and its fragments is in agreement with Kang et al., [17] and identified

as saccharide.

Peaks **10**-**22** were recognized as flavonol glycoside compounds. The fragments of peaks **10**, **11**, **13**, **14** and **16** are characterized for quercetin nucleus, the appearance of peak **10** with pseudomolecular ion at *m/z* 625[M-H]$^-$ and its fragments at *m/z* 463 [M-H-162]$^-$ after the loss of glucose followed by *m/z* 301 [M-H-2×162]$^-$ with another loss of glucose sugar, indicated that the glucoside moieties are in two different positions. In the same manner and in comparison with authentic stander and literature, compound **11** *m/z* 609 was identified as quercetin-3-*O*-glucoside-7-*O*-rhmnaoside [18] while compound **14** *m/z* 771 was identified as quercetin-di-*O*-glucoside-*O*-rhmnaoside. Peak **13** have pseudomolecular ion peak at *m/z* 831[M-H]$^-$, gave fragment ions *m/z* 669 [M-H-162]$^-$ after loss of glucose, *m/z* 463 [M-H-162-206]$^-$ after loss of sinapic acid and *m/z* 301[M-H-162-206-162]$^-$ withdrawing of another glucose suggesting that compound **13** could be assigned as quercetin-*O*-sinapoylglucoside-*O*-glucoside. Peak **16** identified as quercetin-di-*O*-benzoyl glucoside and appears with

TABLE 1. Phenolic compounds identified in 70% methanolic extract of *B. senegalensis* by LC-ESI (-ve)-MS.

Peak no.	t_R (min)	M	[M-H]$^-$	*m/z* fragments	Tentative identification
1	2.84	342	341	179	Caffeic acid glucoside
2	3.26	413	412	332, 259, 241, 97, 96, 75	Glucocapparine sulfate (isomer I)
3	3.92	413	412	332, 259, 241, 97, 96, 75	Glucocapparine sulfate (isomer II)
4	14.78	314	313	191, 147, 85	Benzoylglucarate (isomer I)
5	18.87	314	313	191, 147, 85	Benzoylglucarate (isomer II)
6	19.96	314	313	191, 147, 85	Benzoylglucarate (isomer III)
7	22.63	326	325	185, 163, 119	Coumaric acid glucoside
8	24.47	314	313	191, 147, 85	Benzoylglucarate (isomer IV)
9	29.31	372	371	249, 231, 121, 113	Saccharide
10	31.23	626	625	463, 301	Quercetin-di-*O*- glucoside
11	31.65	610	609	463, 301	Quercetin- *O*- glucoside-*O*-rhmnaoside
12	32.23	640	639	477, 315, 301	Rhamentin-di-*O*-glucoside
13	40.08	832	831	669, 463, 301	Quercetin-*O*-sinapoyl glucoside - *O*-glucoside
14	40.75	772	771	609, 463, 301	Quercetin-di-*O*-glucoside-*O*-rhamnoside
15	41.75	786	785	623, 477, 315	Rhamentin-di-*O*- glucoside-*O*-rhmanoside
16	42.92	834	833	729, 567, 463, 301	Quercetin-di-*O*-benzoyl glucoside
17	43.59	744	743	625, 463,301	Unknown
18	46.68	608	607	299	Rhamnocitrin-*O*-rutinoside
19	47.85	800	799	637, 461, 299	Rhmanocitrin-*O*-feruloyl glucoside *O*-glucoside
20	48.51	770	769	607, 299	Rhmanocitrin- *O*-coumaroyl glucoside -*O*- glucoside
21	50.60	462	461	299	Rhmanocitrin-*O*- glucoside
22	53.36	608	607	461, 299	Rhmanocitrin- *O*-coumaroyl glucoside

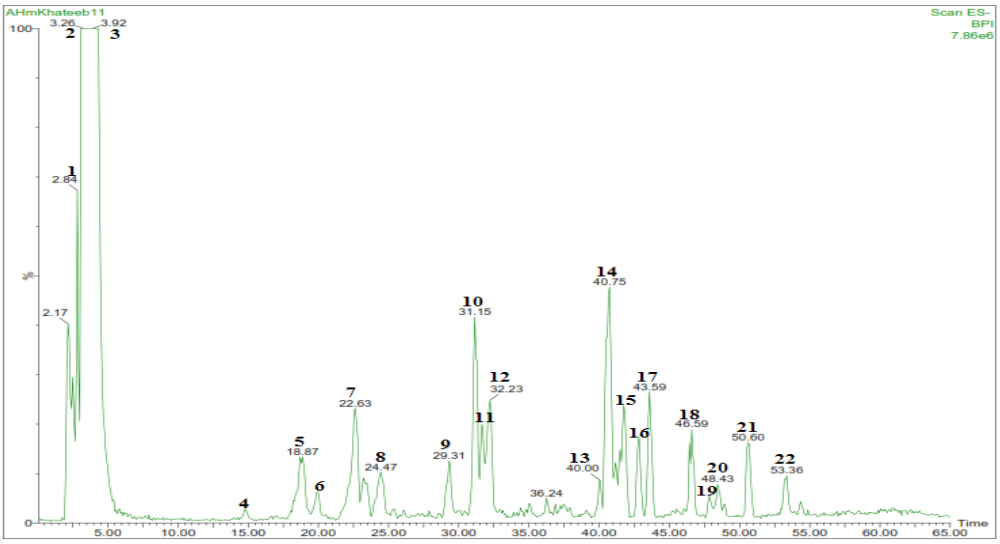

Fig. 1. LC-ESI-MS chromatogram of B. senegalensis .

pseudomolecular ion peak at *m/z* 833[M-H]$^-$ and gave fragment ions *m/z* 729, 567, 463, 301 that is due to the loss of benzoyl group [M-H-102]$^-$ then glucose moiety [M-H-102-162]$^-$ and repeated the same group in the same manner, from our best of knowledge compounds **13** and **16** identified for the first time from nature.

The fragments of Peaks **12**, **15** are characterized for rhamnetin nucleus (*m/z* 315, 301); they appeared at molecular ion peak at *m/z* 639, 785 respectively, the fragmentation of the two peaks indicated that compound **12** is rhamentin-di-*O*-glucoside peak while compound **15** is rhamentin di-*O*- glucoside-*O*-rhmanoside.

Peaks **18 - 22** are characterized for rhamnocitrin nucleus (*m/z* 299), peak 18 is a diglycoside compound with molecular ion peak *m/z* 607, the departure of both sugars with the presence of fragment at *m/z* 299 [M-H-146-162]$^-$ only indicated that they are at the same position in the rhamnocitrin nucleus, while compound **21** is monoglucoside of rhamnocitrin. Peaks **19**, **20** and **22** are rhamnocitrin acylated derivatives which were previously reported from the plant under investigation [8].

In vitro anticancer and antiviral activities.

Plant extracts show interesting anticancer effects on different cell lines yielding higher activity compared with pure natural and synthetic compounds. This remarkable effect is due to synergy between different compounds [19]. Among the important anticancer constituents, phenolic compounds, especially flavonoids. Kampferol, quercetin and their derivatives proved to be effective against numerous cancer cell lines [19]. The main components in the tested extract are phenolic acids and flavonoids.

The aqueous methanolic extract of *B. senegalensis* was tested for cytotoxic activity, (at concentrations between 0, 5, 12.5, 25, 50 µg/ml) against three tumor cell lines: HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT (colon carcinoma cell line) as flavonoids had reported having anticancer and antioxidant activities [20]. Results concluded that the *in vitro* cytotoxicity of the extract showed a moderate antitumor activity against the three cell lines HEPG2 (IC_{50} 24.6 µg/ml), MCF7 (IC_{50} 22.5 µg/ml) and HCT (IC_{50} 40.3 µg/ml).

Antiviral activity of *B. senegalensis* aqueous methanolic extract was measured using plaque reduction assay; it showed 90 % inhibition against H5N1viruses at concentration of 150 µg/ml.

This is considered to be the first study deals with *in vitro* anticancer and antiviral activities of *B. senegalensis* and the results encourage further investigation to isolate and identify the bioactive compounds present in the extract.

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تحليل LC-ESI-MS، الأنشطة المضادة للأورام والفيروسات لمستخلص الميثانول المائى لنبات المخيط

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تم تحديد واحد وعشرين مركبا (اثنتا عشرة فلافونويد وأربعة أيزومرات بنزوويل جلوكارات واثنين من أحماض الفينول واثنين من الجلوكوسينولات وسكاريد واحد) من مستخلص الميثانول المائى لنبات المخيط. من بين هذه المركبات . مركبي ١٣ و ١٦ تم تعريفهم لاول مرة.

كما وجد ان مستخلص نبات المخيط يثبط فيروس H5N1 بنسبة ٪ ٩٠. كما أشارت النتائج الى وجود نشاط متوسط لمستخلص النبات قيد الدراسة لمضادات للأورام ضد ثلاثة من الخلايا السرطانيه HEPG2, MCF7, HCT