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Antimicrobial and cytotoxic potentials of *Buddleja polystachya* extracts

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Abstract

Most of the species of *Buddleja* have found applications in folk medicine. This study aimed to evaluate the *in vitro* antimicrobial and cytotoxic potentials of *B. polystachya* extracts. Four extracts were prepared **A-D** (dichloromethane, ethyl acetate, *n*-butanol, and aqueous extracts, respectively). The antimicrobial activity was evaluated using the broth micro-dilution assay for minimum inhibitory concentrations (MIC). The crystal violet staining method (CVS) was used for the evaluation of the cytotoxic activity on HepG-2, MCF-7 and HCT-116 human cell lines. Results showed that the highest antimicrobial activity was given by the ethyl acetate extract followed by the dichloromethane extract, while the *n*-butanol revealed moderate activity against gram-positive bacteria only with no activity against the rest of tested microorganisms. The aqueous extract was totally ineffective against all tested microorganisms at 20 mg/mL. Among the four extracts tested, dichloromethane and ethyl acetate extracts showed the highest cytotoxic activity on all three human cell lines.

Introduction

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives each year. An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings. Plants, vegetables, herbs and spices that were used in folk and traditional medicine, have been accepted currently as one of the main sources of cancer chemopreventive drug discovery (Sarkar and Mandal, 2011). Additionally, investigation of the antimicrobial properties of plants has brought attention to the opportunity of producing a natural and environment-friendly source that could replace the synthetic antimicrobial compounds (Zhu et al., 2011). With the increase of bacterial resistance to antibiotics, there is considerable interest to investigate the antimicrobial effects of different extracts against a range of bacteria, to develop other classes of natural antimicrobials useful for the infection control (Bakri and Douglas, 2005). Equally important are the dermatophytic infections which are the major causes of morbidity-associated superficial mycoses (Gupta and Cooper, 2008) and are responsible for serious fungal human pathogenic infections that have increased during the last decades (Arif et al., 2011). It was thus deemed of interest to investigate the *in vitro* antimicrobial and cytotoxic potentials of *B. polystachya* organic extracts taking in consideration that most of the species of *Buddleja* have found several applications in folk medicine (Houghton, 1984) and that there is no previous report on the antimicrobial and cytotoxic activities of *B. polystachya*.

Materials and Methods

Plant material

The aerial parts of *B. polystachya* were collected from

Albaha city in Saudi Arabia in June 2011. The plant was identified by Prof. Mohammed Youssef, Department of Pharmacognosy, College of Pharmacy, King Saud University. A voucher specimen (#15270) was deposited at the Department of Pharmacognosy, College of Pharmacy, King Saud University.

Preparation of organic extracts

The air dried powdered plant (1 kg) was extracted with 85% EtOH by cold maceration, till exhaustion. The ethanol extract was dried using rotary evaporator to give a dark residue (120 g), which was suspended in water and subsequently extracted with dichloromethane, ethyl acetate and *n*-butanol. Each fraction was dried over anhydrous sodium sulphate and evaporated to dryness to yield fractions **A** (dichloromethane, 40 g), fraction **B** (ethyl acetate, 10.6 g), fraction **C** (*n*-butanol, 22 g) and fraction **D** (remaining aqueous mother liquor, 47 g). Analytical grade solvents (Sigma-Aldrich, USA), were used for the extraction process.

Determination of antimicrobial activity

Test organisms

In the current study, three gram-positive, six gram-negative as well as four fungi were used to assess the antimicrobial activity: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pyogenes* (ATCC 10782), *Neisseria gonorrhoeae* (ATCC 49226), *Proteous vulgaris* (ATCC 6380), *Klebsiella pneumoniae* (ATCC 31488), *Shigella flexneri* (ATCC 29903), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). The fungi used were *Aspergillus fumigatus* (ATCC 90906), *Candida albicans* (ATCC 10231), *Absidia corymbifera* (ATCC 46774) and *Trichophyton mentagrophytes* (ATCC 9533). The microbial strains were obtained from American Type Culture Collection (ATCC).

Antimicrobial activity

Agar-well diffusion was employed to assess the antimicrobial activity. Mueller-Hinton agar was used as media for bacteria while Sabouraud dextrose agar was used for fungi. Bacterial and fungal suspensions were prepared equal to the turbulence of 0.5 McFarland standard in 100 µL volume and were cultivated on agar medium. Thereafter, 6 mm diameter wells were punched in the used agar plates. Ampicillin, Gentamicin and Amphotericin B (30 µg/mL) were used as standard agents against gram-positive bacteria, gram-negative bacteria and fungi respectively. The bacterial plates were incubated at 37°C, while fungal plates were incubated at 25°C. After incubation, the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone observed. Each test was performed twice and the average of the results was calculated. The extraction solvents were used as negative control (NCCLS, 2002 and NCCLS, 2004).

Determination of the minimum inhibitory concentration (MIC)

The MIC was determined using the broth micro-dilution method (NCCLS, 2002; NCCLS, 2004). Bacteria were grown in Mueller-Hinton broth while fungi were grown in Sabouraud broth. Then, 20 µL of 10^6 cells/mL were inoculated in tubes with broth media supplemented with different concentrations (two fold serial dilutions 1000-0.0015 µL) of samples. After 24 hours at 37°C for bacteria and 3-7 days at 25°C for fungi, the MIC of each sample was measured through optical density in the spectrophotometer (620 nm), non-inoculated broth media served as negative control. All determinations were performed in triplicate.

Cytotoxicity assay

Cell culture

Mammalian cell lines: MCF-7 cells (human breast cancer cell line), HepG2 (human liver cancer cell line), and HCT-116 (human colon cancer cell line) were obtained from VACSERA Tissue Culture Unit. The cells were propagated in Dulbeccos modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St Louis, Mo, USA), 1% L-glutamine, HEPES buffer and 50 µg/mL gentamicin (Sigma Chemical Co., USA). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub-cultured two times a week.

Evaluation of cellular cytotoxicity

The cytotoxic activity was evaluated by the crystal violet staining (CVS) method (Saotome et al., 1989; Itagaki et al., 1991). Briefly, in a 96-well tissue culture microplate, the cells were seeded at a cell concentration of 1×10^4 cells per well in 100 µL of growth medium. Fresh medium containing different concentrations of extracts **A-D** were added after 24 hours of seeding at 37°C. Serial twofold dilutions of the tested extracts were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates using a multi-channel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5%CO_2 for a period of 48 hours. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells was found not to affect the experiment. After the 48 hours incubation period, the viable cells yield was determined by a colorimetric method. In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed and the plates were rinsed using distilled water. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly. The quantitative analysis (colorimetric evaluation of fixed cells) was performed by measuring the absorbance in an automatic Microplate reader (TECAN, Inc.) at 595

nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested extracts. All experiments were carried out in triplicate. The effect on cell growth was calculated as the difference in absorbance percentage in the presence and absence of the tested extracts and illustrated in a dose-response curve. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was obtained from this dose-response curve. The standard antitumor drug used was vinblastine sulfate.

Statistical analysis

Data were expressed as means ± SD. Unpaired student t -test was conducted, using the GraphPad InStat (ISI Software) computer program. Differences were considered significant at P values of less than 0.05.

Results and Discussion

Antimicrobial activity

The antimicrobial activity of the studied extracts was evaluated by determining zone of inhibition and MIC values against three gram-positive, six gram-negative and four fungi (Table I and II).

The antimicrobial activities of the different extracts of *B. polystachya* varied. The highest antimicrobial activity was shown by the ethyl acetate extract (15.3-20.3 mm diameter of zone of inhibition), followed by the dichloromethane extract (11.4-19.3 mm diameter of zone of inhibition), while the *n*-butanol extract revealed moderate to low activity against gram-positive bacteria only with no activity against the rest of tested microorganisms. The aqueous extract was totally ineffective against all tested microorganisms at 20 mg/mL. The MIC of *B. polystachya* extracts ranged from 1000-3.9 µg/mL, with the ethyl acetate extract demonstra-ting the lowest values (3.9 µg/mL) against *Absidia corymbifera* (ATCC 46774), *Trichophyton mentagrophytes* (ATCC 9533) and *Klebsiella pneumoniae* (ATCC 31488) followed by the dichloromethane extract against *Trichophyton mentagrophytes* (ATCC 9533) and *Klebsiella pneumoniae* (ATCC 31488) with MIC value 7.8 µg/mL (Table II). Constituents present in *Buddleja* species may display antimicrobial activity if present in sufficient concentrations. Verbascoside, the major phenylethanoid from the leaves of *B. globosa* has been shown to be the dominant antibacterial compound when tested against *Staphylococcus aureus* and *E. coli* (Pardo, 1993). A study has shown that the triterpenoid buddlejasaponin 1 has activity against 9 yeast species (Emam et al., 1997). More recently, buddledin A and buddledin B have been shown to be selectively active against various dermatophytic fungal species (Mensah et al., 2000).

The test organisms used in this study are associated with various forms of human infections. From a clinical

Table I

Mean zone of inhibition beyond 6 mm well diameter using (20 mg/mL) concentration of A-D

Tested microorganisms	Mean zone of inhibition in mm ± SD				
Fungi	A	B	C	D	Amphotericin B
Aspergillus fumigatus (ATCC 90906)	14.6 ± 0.6	18.2 ± 0.3	NA	NA	23.7 ± 0.1
Candida albicans (ATCC 10231)	11.4 ± 0.4	15.3 ± 0.4	NA	NA	21.9 ± 0.1
Absidia corymbifera (ATCC 46774)	17.8 ± 0.4	19.8 ± 0.4	NA	NA	26.4 ± 0.2
Trichophyton mentagrophytes (ATCC 9533)	19.3 ± 0.4	20.3 ± 0.3	NA	NA	25.4 ± 0.2
Gram-positive bacteria					Ampicillin
Staphylococcus aureus (ATCC 25923)	14.2 ± 0.3	17.4 ± 0.4	11.6 ± 0.4	NA	28.9 ± 0.1
Staphylococcus epidermidis)ATCC 12228)	16.8 ± 0.4	18.6 ± 0.4	12.0 ± 0.4	NA	25.4 ± 0.2
Streptococcus pyogenes (ATCC 10782)	17.1 ± 0.3	15.6 ± 0.4	13.0 ± 0.4	NA	26.4 ± 0.3
Gram-negative bacteria					Gentamicin
Neisseria gonorrhoeae (ATCC 49226)	13.6 ± 0.4	17.0 ± 0.4	NA	NA	19.9 ± 0.2
Proteous vulgaris (ATCC 6380)	15.9 ± 0.6	16.8 ± 0.4	NA	NA	23.4 ± 0.3
Klebsiella pneumoniae (ATCC 31488)	18.6 ± 0.6	20.2 ± 0.3	NA	NA	26.3 ± 0.2
Shigella flexneri (ATCC 29903)	14.6 ± 0.4	17.6 ± 0.3	NA	NA	24.8± 0.2
Pseudomonas aeruginosa (ATCC 27853)	12.3 ± 0.4	15.3 ± 0.6	NA	NA	17.3 ± 0.1
Escherichia coli (ATCC 25922)	15.9 ± 0.2	16.3 ± 0.6	NA	NA	25.3 ± 0.2

*NA: No activity, data are expressed in the form of mean ± SD

Table II

MICS (µg/mL) of A-D against tested microorganisms

Tested microorganisms	Sample MIC				
Fungi	A	B	C	D	Amphotericin B
Aspergillus fumigatus (ATCC 90906)	125	15.6	NA	NA	1.0
Candida albicans (ATCC 10231)	1000	125	NA	NA	3.9
Absidia corymbifera (ATCC 46774)	15.6	3.9	NA	NA	0.1
Trichophyton mentagrophytes (ATCC 9533)	7.8	3.9	NA	NA	0.1
Gram positive bacteria					Ampicillin
Staphylococcus aureus (ATCC 25923)	250	62.5	1000	NA	0.0
Staphylococcus epidermidis (ATCC 12228)	31.3	15.63	500	NA	0.1
Streptococcus pyogenes (ATCC 10782)	31.3	125	250	NA	0.1
Gram negative bacteria					Gentamicin
Neisseria gonorrhoeae (ATCC 49226)	250	31.3	NA	NA	7.8
Proteous vulgaris (ATCC 6380)	62.5	31.3	NA	NA	1.0
Klebsiella pneumoniae (ATCC 31488)	7.8	3.9	NA	NA	0.1
Shigella flexneri (ATCC 29903)	125	15.6	NA	NA	0.2
Pseudomonas aeruginosa (ATCC 27853)	500	125	NA	NA	62.5
Escherichia coli (ATCC 25922)	62.5	62.5	NA	NA	0.1

NA: no activity

point of view, *Klebsiella pneumoniae* is the most important member of the *Klebsiella* genus of Enterobacteriaceae and it is emerging as an important cause of neonatal nosocomial infection (Gupta et al., 1993). The majority of nosocomial fungal infections are caused by *Candida* species, with *Candida albicans* being the most common etiological agent of fungal bloodstream infections. Together with *Candida* infections, *Aspergillus* infections, which mostly affect granulocytopenic and other immunocompromised patients, account for 90% of all nosocomial fungal infection. While, *Absidia corymbifera* is intrinsically resistant to the available antifungal drugs, and is becoming increasingly recognized as a source of deep fungal infections (Fridkin and Jarvis, 1996).

The demonstration of activity against both gram-negative, Gram positive bacteria and fungi is an indication that *B. polystachya* ethyl acetate and dichloromethane extracts can be a source of bioactive substances that could be of broad spectrum activity. Furthermore, they can be a source of very potent antibiotic substances that can be used against drug resistant microorganisms prevalent in hospital environments.

Cytotoxic activity

The cytotoxic activity of **A-D** against MCF-7, HepG-2 and HCT-116 carcinoma cell lines, was determined using CVS method employing vinblastine as a reference drug. The response parameter (IC_{50}) was calculated for each cell line (Table IIIV).

From the results shown, it could be observed that all extracts possessed a dose dependent cytotoxic effect against all three cell lines. However, dichloromethane (**A**) and ethyl acetate (**B**) extracts showed the highest cytotoxic activities. They exhibited more selective cytotoxic activity against MCF-7 (IC_{50} = 10.2 and 6.8 µg/ml, respectively) and HepG-2 (IC_{50} = 6.1 and 7.3 µg/mL, respectively) cell lines than on the HCT-116 cell line, compared to vinblastine sulphate as reference drug (IC_{50} = 4.6 and 4.6 µg/mL, respectively). The lowest cytotoxic activity was shown by *n*-butanol and the aqueous extracts against the three cell lines. The aqueous extract was more selective on HepG-2 and HCT-116 cell lines (IC_{50} = 22.1 and 24.7 µg/mL, respectively). Thus, both the dichloromethane and ethyl acetate extracts of *B. polystachya* could be further investi-gated phytochemically to isolate promising cytotoxic compounds. Verbascoside, a phenylethanoid was isolated from the flowers of *B. officinalis* and was proved to have anti-cancer activities (Jiaoshe et al., 1997).

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Table III

***In vitro* cytotoxic activities of A-D on MCF-7**

Tumor cell line MCF-7	Vinblastine sulfate	Mean of surviving fraction ± SD#			
Sample concentration (µg/mL)		A	B	C	D
50	7.8 ± 1.0	17.2 ± 4.1[c]	13.8 ± 1.0[b]	46.0 ± 1.7[a]	24.2 ± 2.7[a]
25	15.2 ± 1.2	26.7 ± 2.7[b]	25.2 ± 2.1[b]	80.5 ± 1.0[a]	67.9 ± 2.3[a]
12.5	29.3 ± 2.7	38.4 ± 3.6[c]	35.0 ± 2.6	91.8 ± 0.98[a]	85.1 ± 1.8[a]
6.25	42.4 ± 2.2	70.7 ± 3.1[a]	51.3 ± 3.5[c]	97.9 ± 0.4[a]	96.8 ± 0.4[a]
3.125	56.5 ± 2.0	85.0 ± 1.3[a]	69.6 ± 2.0[a]	100[a]	100[a]
1.56	67.2 ± 2.9	91.7 ± 0.7[a]	85.1 ± 2.2[b]	100[a]	100[a]
*IC_{50} (µg/mL)	4.6	10.2	6.8	47.1	35.2

#Mean of surviving fraction ± standard deviation: Mean of three assays. [a]p<0.001, [b]p<0.01, p<0.05 compared to reference drug. *IC_{50}: Concentration of extract required to reduce cell survival by 50%

Table IV

***In vitro* cytotoxic activities of A-D on HepG-2**

Tumor cell line HepG2	Vinblastine sulfate	Mean of surviving fraction ± SD#			
Sample concentration (µg/mL)		A	B	C	D
50	14.4 ± 1.4	9.8 ± 2.9	8.8 ± 1.7[c]	47.1 ± 1.9[a]	19.7 ± 3.0
25	16.1 ± 2.2	20.9 ± 3.2	19.7 ± 1.3	62.4 ± 3.5[a]	45.9 ± 3.7[a]
12.5	24.3 ± 3.0	31.3 ± 4.2	30.6 ± 2.3[c]	85.9 ± 1.3[a]	63.9 ± 2.8[a]
6.25	45.1 ± 2.0	48.7 ± 3.5	53.8 ± 2.1[b]	94.3 ± 1.0[a]	82.4 ± 1.1[a]
3.125	55.0 ± 2.3	69.4 ± 1.9[b]	75.4 ± 2.0[a]	98.9 ± 0.3[a]	91.5 ± 1.3[a]
1.56	72.1 ± 3.1	85.2 ± 1.7[b]	89.7 ± 1.4[a]	100[a]	96.9 ± 0.7[a]
*IC_{50} (µg/mL)	4.6	6.1	7.3	45.3	22.1

#Mean of surviving fraction ± standard deviation: Mean of three assays. [a]p<0.001, [b]p<0.01, [c]p<0.05 compared to reference drug. *IC_{50}: Concentration of extract required to reduce cell survival by 50%

Table V

***In vitro* cytotoxic activities of A-D on HCT-116**

Tumor cell line HCT-116	Vinblastine sulfate	Mean of surviving fraction ± SD#			
Sample concentration (µg/mL)		A	B	C	D
50	16.3± 1.1	22.0 ± 3.2[c]	16.4 ± 4.2	48.5 ± 3.5[a]	36.9 ± 1.7[a]
25	21.7 ± 2.4	38.7 ± 2.0[a]	39.5 ± 4.3[b]	71.8 ± 1.9[a]	49.5 ± 1.0[a]
12.5	28.2 ± 4.6	59.3 ± 3.1[a]	63.5 ± 4.3[a]	86.7 ± 1.0[a]	68.8 ± 1.3[a]
6.25	38.1 ± 5.3	75.0 ± 2.3[a]	78.1 ± 4.0[a]	96.3 ± 0.4[a]	84.4 ± 0.9[a]
3.125	47.5 ± 4.0	81.6 ± 1.8[a]	90.6 ± 2.5[a]	100[a]	92.3 ± 1.1[a]
1.56	53.4 ± 4.0	92.6 ± 0.9[a]	98.1 ± 0.8[a]	100[a]	96.9 ± 0.8[a]
*IC_{50} (µg/mL)	2.6	18.1	19.5	48.4	24.7

#Mean of surviving fraction ± standard deviation: Mean of three assays. [a]p<0.001, [b]p<0.01, [c]p<0.05 compared to reference drug. *IC_{50}: Concentration of extract required to reduce cell survival by 50%

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