



Original Article

Total phenolic content, antioxidant and antimicrobial activities of *Blepharis edulis* extracts

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Abstract

Blepharis edulis is traditionally used as an antiseptic, purgative, aphrodisiac and anti-inflammatory agent. The extracts of plant aerial parts were screened for total phenolic content (TPC) gallic acid equivalents (GAE), total flavonoid compound (TFC) quercetin equivalents (QE), antioxidant capacity and its antimicrobial activity by micro broth dilution assay. The 50%-inhibition values of BHT and 70% (v/v) aqueous ethanol, 70% (v/v) aqueous methanol, methanol, and water extracts of *B. edulis* according to the DPPH method were found to be 19.6, 71.2, 73.7, 81.4, and 218.4 µg/ml, respectively. TPC ranged from 38.9 to 102.7 mg GAE/g dry extracts. The antimicrobial activity showed that yeast and fungi were sensitive and resistant microorganisms to the extracts. The 70%-methanol extract showed more drastic antimicrobial activity than the others. The antimicrobial activity of ethanolic extract is the same as of the methanolic extract; water extract had the weakest antimicrobial activity.

Keywords: *Blepharis persica*, *Blepharis edulis*, phenolic total content, antimicrobial activity, antioxidant activity

1. Introduction

Nowadays, essential oils and various plant extracts have received a renewed attention as antimicrobial, antifungal, and antioxidant compounds and have formed the basis of many applications in food preservation, pharmaceuticals, and industries (Lis-Balchin and Deans, 1997). Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibition the initiation or propagation of oxidizing chain reactions. Oxidants and oxidized reactions are considered as major contributors to the induction and/or progress of many pathological conditions, such as cancer, chronic inflammation, Alzheimer's, Parkinson's and heart diseases. Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects. Phenolic compounds, with

the potential ability for losing hydrogen atoms and/or single electrons (due to the stability of resulting free radicals), and with metal chelating properties (due to the presence of hydroxyl and carbonyl functional groups in their structures) exhibit considerable antioxidant activities (Rice-Evans *et al.*, 1996).

Food spoilage is one of the most important issues of food industries and food borne diseases is a global concern even in developed countries. Food deterioration is predominantly caused by the growth of microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* sp, *Candida* sp, and *Aspergillus* sp. For these reasons, the search on plant extracts and evaluation of their biological activities are undoubtedly very important. Natural aromatic plants have constructed new research categories for developing new antimicrobial and antioxidant foods (Kordali *et al.*, 2005).

Blepharis genus of Acanthaceae family comprises approximately 100 species with a tropical and subtropical distribution. Only one of them is endemic, namely *B. persica* (syn. *B. edulis*) (Persian name: kharsonbol), which grows in

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the south of Iran (Mozaffarian, 1996). *B. edulis* is a small, perennial plant that inhabits in deserts of the Middle East and Africa (Gutterman, 1972). In the ethnomedicinal literature of India, *B. edulis* is used as food to increase sperm count and as aphrodisiac plant (Pande and Pathak, 2009). In Thai traditional medicine, the plant is used as a purgative and as anti-inflammatory agent, as well as the leaves dispensed with pepper (*Piper nigrum* L.) as tonic pills for long life (Kanchanapoom *et al.*, 2001). According to our knowledge some of the species belonging to *Blepharis* genus have been previously investigated from different points of view: identification of vebascoside and isovebascosides in *B. aspera* and blepharin in *B. edulis* (Pratt *et al.*, 1995; Mmatli *et al.*, 2007), total phenolic content and antioxidant capacity of *B. edulis* seeds (Surveswaran *et al.*, 2007), antimicrobial activities of *B. ciliaris* and *B. edulis* (Harraz *et al.*, 1996; Keymanesh *et al.*, 2009), *B. repens*, for the treatment of aphthae and toothache and effectiveness of *B. edulis* seeds in libido subjects (Hebbar *et al.*, 2004; Pand and Pathak, 2009) and in Tanzanian traditional medicine, decoction of *B. panduriformis* Lindau for treatment of various infectious diseases, such as dysentery diarrhea (Maregesi *et al.*, 2007). Available literatures indicate no previous study on antioxidant and antimicrobial properties, TPC and TFC assays for different extracts of Iranian *Blepharis* aerial parts. The objectives of this study was the evaluation of TPC, TFC, antioxidant capacity of *B. edulis* extracts and its antimicrobial activities against different kinds of microorganisms *in vitro* condition.

2. Material and Methods

2.1 Plant material

B. edulis whole plant was collected from Geno Mountain (Dargir Village, Bandar Abbas, Iran) in April 2010 by the Agricultural Department, Research Center of Barij Essence and identified and authenticated under number 193-1.

2.2 Preparation of *B. edulis* extracts

Extraction was performed with water, methanol, methanol-water (70:30, v/v) and ethanol-water (70:30, v/v). The powdered dried *B. edulis* aerial parts were mixed with solvent at the ratio of 1:10 (w/v) for 6 hrs at ambient temperature. Then, the mixture was filtered through Whatman filter paper No. 2; the residue rinsed with the same solvent and the extract dried under vacuum. The yield of each extract was calculated.

2.3 Total phenolic content

Total phenolic contents (TPC) of crude extracts were determined by a spectrophotometer (Perkin-Elmer Lambda EZ-210 UV/VIS), with dual-beam, using the Folin-Ciocalteu's reagent (Haghi *et al.*, 2011). Each dry extract (10 mg) was

dissolved in 10 ml of itself solvent (1 mg/ml). 0.2 ml of extract was transferred into a 5 ml volumetric flask and swirled with 3 ml of deionized water. 0.25 ml of Folin-Ciocalteu's reagent was added and swirled. After 3 min, 0.75 ml of 20% (w/v) sodium carbonate solution was added and mixed. This was recorded as time zero. Deionized water was added to make up the volume to 5 ml exactly. The solution was mixed thoroughly and allowed to stand at ambient temperature for 2 hrs until the characteristic blue color developed. The absorbance of reaction mixture was measured at 760 nm. Quantification of TPC was based on a standard curve generated with GA at 760 nm using the following equation: $Abs = 0.0054w + 0.015$, where Abs is absorbance and w is the weight (μg). All tests were conducted in triplicate and averaged. The results were expressed as mg of TPC per 100 mg of dry extract as gallic acid equivalents (GAE).

2.4 Total flavonoid content

The aluminum chloride colorimetric method was used to estimate the total flavonoid content (TFC) in crude extracts (Chang *et al.*, 2002). 50 mg of dry extract was dissolved in 10 ml of itself solvent (5 mg/ml). 0.5 ml of extract was mixed with 2 ml of appropriate solvent, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and diluted to the mark with distillate water in a 5 ml volumetric flask. The absorbance was measured at 415 nm after 30 min. TFC in the extracts was determined using a standard curve established with quercetin (25-100 μg/ml) and the results expressed as mg of QE per mg of dry extract.

2.5 Antioxidant activity

2.5.1 Free radical-scavenging activity by DPPH method

The hydrogen atom or electron donation ability of the extracts was measured from the bleaching of purple colored methanol solution of DPPH (Sarker *et al.*, 2006). A stock solution of each extract (1 mg/ml) was prepared in itself solvent and diluted with methanol in the range of concentrations 30 to 300 μg/ml. The extracts diluted (2 ml) were added to 2 ml of freshly prepared DPPH methanol solution (60 μg/ml) and mixed. Decreasing of absorbance of tested samples was monitored every 10 min at 517 nm using a UV-Vis spectrophotometer (Perkin Elmer EZ-210) and continued for 70 min until the reaction reached a plateau. Inhibition of free radical DPPH in percent (I%) was calculated as follow, $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and sample is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means. BHT as the reference antioxidant was prepared in methanol. Dilutions were made to obtain concentrations ranging from 10 to

60 µg/ml. Diluted solutions were tested as described above to establish DPPH scavenging capacity standard curve.

2.5.2 β -carotene/linoleic acid bleaching test

The β -carotene bleaching activity of extracts was performed as given elsewhere (Wettasinghe and Shahidi, 1999). 5 mg of β -carotene was dissolved in 10 ml chloroform and 3.0 ml of this solution was pipette into a 100 ml round bottom flask. Chloroform was removed using a rotary evaporator under vacuum at 40°C, and then 25 µl of linoleic acid, 200 µl of Tween 80 and 50 ml of aerated distilled water were added to the flask with vigorous shaking. The emulsion (4.8 ml) was added to a tube containing 0.2 ml of the extract (5 mg/ml) and the absorbance immediately measured at 470 nm against a blank as zero time. A blank sample, devoid of β -carotene, was prepared for background subtraction. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored by measuring absorbance at 470 nm over a 120 min-period. The antioxidant property (inhibition percentage, I%) of the samples was determined using the following equation, $I\% = (A_{\beta\text{-carotene}} \text{ after 2 hrs assay} / A_{\text{initial } \beta\text{-carotene}}) \times 100$, where $A_{\beta\text{-carotene}}$ after 2 hrs assay is the absorbance of β -carotene after 2 hrs assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of β -carotene at the beginning of the experiments. BHT was used as positive control.

2.6 Microbial strains

Staphylococcus aureus ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermidis* ATCC 14490, *Enterococcus faecium* ATCC 25778, clinical isolate of *Streptococcus agalactiae*, *Bacillus cereus* ATCC 1247, *Bacillus subtilis* ATCC6051, *Streptococcus pyogenes* ATCC 8668, *Staphylococcus saprophyticus* ATCC 15305, *Klebsiella pneumoniae* ATCC 1053, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Shigella dysenteriae* PTCC1188, *Proteus vulgaris* ATCC1079, *Streptococcus sanjuis* ATCC 10556, *Streptococcus salivarius* ATCC 9222, *Enterobacter aerogenes* NCTC 10009, *Pseudomonas aeruginosa* ATCC 9027, and fungi *Candida albicans* ATCC 10231, *Aspergillus flavus*, *Aspergillus niger* ATCC 16404, *Aspergillus parasiticus* ATCC 15517 were used. Bacterial suspensions were made in brain heart infusion (BHI) broth to a concentration of approximately 10^8 CFU/ml using standard routine spectrophotometric methods. Suspensions of fungi were made in Sabouraud dextrose broth. Subsequent dilutions were made from the above suspensions, which were then used in the tests.

2.7 Evaluation of antimicrobial activity

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of extracts were determined by micro broth dilution assay. The extracts were

twofold serially diluted with 10% DMSO which contains 100-0.2 mg/ml of each extract. These dilutions were prepared in a 96-well microtitre plate. MOPS-buffered RPMI 1640 (fungi) (Marchetti *et al.*, 2000), cation adjusted Muller Hinton broth (non-fastidious bacteria) (NCCLS, 2006) and Todd Hewitt broth (fastidious bacteria) (Carson *et al.*, 1995) were used as broth media. After shaking, 100 µl of each extract dilutions was added to each well. The above microbial suspensions was diluted (1×10^6 CFU/ml for bacteria; 10^4 for fungi) and then 100 µl was added to each well and incubated at 35°C. MICs were defined as the lowest concentration of extract dilutions that inhibits bacteria and fungi after 24, 48 hrs, respectively. MLC values were the first well that showing no growth on solid media.

2.8 Data analysis

The results of analysis were expressed as the means of three independent analyses. The results of antimicrobial activity was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparison. The level of significance was set at 95%. Statistical analysis was performed with statistical software SPSS 17 (SPSS for Windows; SPSS Inc, Chicago, IL).

3. Results

The extraction yield, TPC, TFC, and IC_{50} of *B. edulis* different extracts are shown in Table 1. The extracts yield varied from 7.3 to 10.3% (w/w) in dried material. Among all tested extracts, the highest yield was obtained from the aqueous extract. On the other hand, efficient extraction with increase solvent polarity was higher than that of non-polar solvent. The TPC and TFC in the extracts were in the range of 38.9 to 102.7 mg/g GAE and 6.2 to 18.7 mg/g QE in dry extract, respectively. The highest content of TPC was in accordance with the results of antioxidant function determination found in the extracts.

3.1 Antioxidant activity

Two complementary test systems were applied to evaluate the antioxidant capacities of the extracts. The results are given in Table 1. In the DPPH test system, extracts shows similar trend with the result of TPC and TFC, indicating that DPPH radical scavenging activity of *B. edulis* extracts is highly related to total phenolic compounds, especially present flavonoid in the extracts. Total antioxidant capacity found by this method was in following order: 70% EtOH > 70% MeOH > MeOH > aqueous extract. The antioxidant activities were compared with BHT with $IC_{50} = 19.61$ µg/ml. The radical-scavenging activity of 70% EtOH extract was equal to about third of that of BHT. In the β -carotene/linoleic acid test, the extracts showed inhibition percent between 10.7 to 33.8% whereas inhibition for 20 ppm BHT was 37.9%.

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity of the extracts.

Solvent of extraction	Yield (%), w/w	TPC (mg/g)	TFC (mg/g)	DPPH ^a IC ₅₀ (μg/ml)	BCB ^b (I%)
water	10.3	38.9	6.2	218.4	10.7
70% MeOH	7.9	100.2	17.2	73.7	31.2
70% EtOH	7.3	102.7	18.7	71.2	33.8
MeOH	6.7	88.7	11.1	81.4	28.5

a:1,1-diphenyl-2-picrylhydrazyl; b: β-carotene/linoleic acid bleaching

3.2 Antimicrobial activity

The extracts obtained from the aerial parts of *B. edulis* were tested against a set of microorganisms in order to estimate their antimicrobial potentials. The strains are different in susceptibility to *B. edulis* extracts. The *B. edulis* extracts have different antimicrobial activity against different kind of microorganisms. The strains and extracts did not have any reciprocal effect (Table 2). Because of different susceptibility of strains to the extracts, we determined the difference between strains (Table 3). *C. albicans*, *B. cereus*, *B. subtilis*, *S. pyogenes* and *Sh. dysenteriae* is the most sensitive microorganisms to this extract with the means of 10.3-13.3 mg/ml for *B. edulis* extract. *A. niger*, *A. parasiticus* and *A. flavus* were less sensitive microorganisms. Means of minimal concentrations of different extracts and antibiotic in homogenous subset exhibited four different subsets. Antibiotics had the smallest value for means of MICs (3.35 μg/ml), followed by 70% methanol extract (15.9 mg/ml). Methanol extract (19.6 mg/ml) and 70% ethanol extract (20.7 mg/ml) has the same antimicrobial activity. The aqueous extract of *B. edulis* had the less antimicrobial activity than the other extracts (42.2 mg/ml) (Table 4).

4. Discussion

Application of medicinal plants as food, preservatives, and drugs is mainly due their biological potentials such

as antioxidant or antimicrobial activities (Bravo, 1998). Solvents have significant effects on *B. edulis* extraction yield. Water extract showed highest yield of dry extract. The TPC, TFC in ethanol and methanol aqueous extracts is higher than that of methanol extract, as the increase in TPC or TFC is related to the increase in *B. edulis* antioxidant activity, while this relation is not between its antimicrobial activity and TPC or TFC. It is reported that the antimicrobial activity of phenolic compound are due to iron deprivation or hydrogen bonding with vital protein such as microbial enzymes (Scalbert, 1991). Other research exhibited that *B. edulis* ethanolic extract (80% V/V) has shown no acceptable antimicrobial activity against bacteria and fungi and the MIC values of *B. edulis* against *S. aureus*, *B. subtilis*, *E. faecalis*, *P. aeruginosa*, *E. coli* and *A. niger* were 1,333, 333, 666, 1,333, 1,333, and 583 μg/ml, respectively (Keymanesh *et al.*, 2009). In this study, 70% methanol extract has shown the best antimicrobial effect compared to ethanolic and methanol extracts. This effect is related to kind of microorganisms and amount of effective antimicrobial compound in the extracts. Flavonoids possess antimicrobial activity against *C. albicans* (Harborne and Williams, 2000), *Aspergillus flavus* (Zheng *et al.*, 1996), *A. tamari*, *A. flavus*, *Cladosporium shaerospermum*, *Penicillium digitatum*, *Penicillium italicum* (Afolayan and Meyer, 1997), HIV-1 (Li *et al.*, 2000), Herpes simplex Virus, poliovirus, Sindbis virus (Selway, 1986), *Salmonella typhimurium* (Dastidar *et al.*, 2004), *Shigella* sp (Vijaya and Ananthan, 1996). There is a relationship between antimicro-

Table 2. Results of analysis between subjects.

Source	Type III sum of squares	df	Mean of Square	F	Sig
Strain	171740857	21	817.8	45.3	0.000
Extracts	69301.6	4	17325.4	959.7	0.000
Minimal	5579.4	1	5579.4	309.1	0.000
Strain*Extract	30131.3	84	358.7	19.9	0.000
Error	5921.5	328	18.1		
Total	309052.2	439			

R squared=0.954 (adjusted R squared=0.938); Strain (microorganisms); Minimal (MIC and MBC); Strain*Extract (reciprocal effect between strain and extract).

Table 3. Sensitivity of microbial strains to *B. edulis* extracts.

	Strains	N	Subsets (μl/ml)
Tukey HSD ^{a,b,c}	<i>C. albicans</i>	20	10.3 ^a
	<i>B. cereus</i>	20	10.9 ^{a,b}
	<i>B. subtilis</i>	20	13.1 ^{a,b,c}
	<i>S. pyogenes</i>	20	13.2 ^{a,b,c}
	<i>Sh. dysenteriae</i>	20	13.3 ^{a,b,c,d}
	<i>S. saprophyticus</i>	20	15.3 ^{b,c,d,e}
	<i>P. vulgaris</i>	19	17.4 ^{c,d,e,f}
	<i>S. sanjuis</i>	20	18.1 ^{d,e,f,g}
	<i>S. salivarius</i>	20	18.9 ^{e,f,g}
	<i>S. aureus</i>	20	19.7 ^{e,f,g}
	<i>S. epidermidis</i>	20	19.9 ^{e,f,g}
	<i>S. agalactiae</i>	20	19.9 ^{e,f,g}
	<i>E. coli</i>	20	21.3 ^{f,g,h}
	<i>K. pneumoniae</i>	20	21.4 ^{f,g,h}
	<i>E. faecium</i>	20	21.4 ^{f,g,h}
	<i>S. typhimurium</i>	20	21.6 ^{f,g,h}
	<i>P. aeruginosa</i>	20	22.9 ^{g,h,i}
	<i>E. faecalis</i>	20	26.1 ^{h,i}
	<i>E. aeruginosa</i>	20	26.9 ^{i,j}
	<i>A. niger</i>	20	31.3 ^{j,k}
	<i>A. parasiticus</i>	20	32.0 ^K
	<i>A. flavus</i>	20	32.2 ^K

N= number of replicates

Table 4. Comparison between different extracts of *B. edulis* and antibiotics.

Extract	Means ± S.E
Antibiotics	3.35 ^a
Methanolic Extract (70)	15.9 ^b
Methanolic Extract (100)	19.6 ^c
Ethanolic Extract (70)	20.7 ^c
Water Extract	42.2 ^d

alpha = 0.05

bial activity of flavonoids and their structures (Sato *et al.*, 1996). The antimicrobial mechanisms of various flavonoids are related to the inhibition of nucleic acid synthesis (Ohemeng *et al.*, 1993), the inhibition of cytoplasmic membrane function (Tsuchiya and Iinuma, 2000), and to the inhibition of energy metabolism (Haraguchi *et al.*, 1996). So, the flavonoids structure in each extract and the microbial difference may affect on its antimicrobial activity.

5. Conclusion

The extracting solvents with different polarities have an effect on extract yield, the total phenolic content, and antioxidant and antimicrobial activities. A direct correlation could

be found between the total phenolic content and antioxidant activity of the extracts. The 70% methanol-extract was found to have the best antimicrobial activity. Different bacteria or fungi showed varied susceptibility to extracts.

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