Antibacterial, antioxidant, anti-cholinesterase potential and flavonol glycosides of *Biscutella raphanifolia* (Brassicaceae)

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**Abstract:** Different extracts of the aerial parts of *Biscutella raphanifolia* (Brassicaceae), which has not been the subject of any study, were screened for the phytochemical content, anti-microbial, antioxidant and anti-cholinesterase activities. We used four methods to identify the antioxidant activity namely, ABTS⁺⁺, DPPH scavening, CUPRAC and ferrous ions chelating methods. Since there is a relationship between antioxidants and cholinesterase enzyme inhibitors, we used two methods to determine the in vitro anti-cholinesterase activity by the use of the basic enzymes that occur in causing Alzheimer’s disease: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The extracts were also tested in vitro antimicrobial activity against various bacteria. The phytochemical study of *B. raphanifolia* afforded four flavonol glycosides; namely, quercetin-3-O-β-D-glucoside, quercetin-3-O-[β-D-glucosyl(1→2)-O-β-D-glucoside], quercetin-3-O-[β-D-glucosyl(1→3)-O-β-D-glucoside] and kaempferol-3-O-[β-D-glucosyl(1→2)-[6″-p-coumaroyl]-β-D-glucoside], being isolated here for the first time from *Biscutella raphanifolia* and the genus. The ethyl acetate extract showed the highest activity in ABTS⁺⁺, DPPH and CUPRAC assays, while the petroleum ether extract demonstrated optimum efficiency metal chelating activity. The dichloromethane and petroleum ether extracts showed a mild inhibition against AChE and BChE. However, the petroleum ether extract showed a good antibacterial activity against the pathovars *Enteropathogenic E. coli* (EPEC), *Enterotoxigenic E. coli* (ETEC) and *Enterococcus faecalis*, whereas the *Enterohemorrhagic E. coli* (EHEC) strain was more sensitive to dichloromethane and n-butanol extracts.

**Keywords:** *Biscutella raphanifolia*, antioxidant, anti-cholinesterase, anti-bacterial, flavonols.

**INTRODUCTION**

For a long time, plants have been the subject of extensive research, heightened by the hope of therapeutically active molecules. The therapeutic use of plants is very old and is experiencing a revival. It is possible to use the whole plant or mining products they provide (Mark et al., 2001). According to the WHO, nearly 6377 species of plants are used in Africa; more than 400 are medicinal plants that constitute 90% of traditional medicine. In 2004, nearly 75% of Africa’s population has used plants to treat and has no access to modern drugs. Today, it has been estimated that the active ingredients from plants represent 25% of prescription drugs for a total of 120 composed of natural origin from 90 different plants (Potterats, 1997). Algeria has a considerable floristic richness. There are about 3000 species of plants of which 15% are endemic (Quezel et al., 1963).

Plants products received great interest as safe antioxidants, since the synthetic antioxidants were reported that they may be accountant for carcinogenesis and damage to the liver (Grice, 1988). Natural compounds are also important where they prevent the oxidative stress damage (Scalbert et al., 2005). Moreover, the delay of evolution of the disease of Alzheimer and the reduction of neuronal degeneration can occur by using of antioxidants (Atta-ur-Rahman et al., 2001). There is a hypothesis that the lack of the necessary amount of neuroregulator acetylcholine is the cause of Alzheimer’s (Grossberg, 2003). Therefore, the treatment of Alzheimer’s disease is effected by the acetylcholinesterase inhibitors drugs. The use of products of natural origin, possessing different biological activities such as anti-cholinesterase, antimicrobial and antioxidant, is more effective than the use of drugs, exhibiting some side effects.

In continuation of our works on plants growing at Constantine, possessing a high antioxidant potential (Bencharif-Betina et al., 2012; Benkiki et al., 2003; Do Rego et al., 2007: Kabouche et al., 2005 and 2007; Kolak et al., 2009; Laggonne et al., 2011; Lakhal et al., 2011 and 2014; Touafek et al., 2011 and 2012), we describe here the phytochemical study of the Algerian species *Biscutella raphanifolia* (Brassicaceae) (Quezel et al., 1963) and its anti-cholinesterase, antibacterial, and antioxidant activities. In this study, we compared the anti-cholinesterase activity with galantamine and the antioxidant activity with different commercial products.

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MATERIALS AND METHODS

General experimental procedures
UV spectra have been recorded on an Agilent 8453, UV-Visible spectrophotometer, in MeOH as blank. Anticholinesterase and antioxidant activities were performed on a Spectravmax340PC 96 well plate microplate reader. Column chromatography (CC): Polyamide (ICN Biomedicals GMBH 09602 and ICN Biomedicals GMBH 09603), Merck silica gel 60 (Merck, Art. 9385), TLC: silica gel 60 F 254 (Art. 5554). 1,1-diphenyl-2-picrylhydrazyl (DPPH), butyldedhydroxyl anisole (BHA), α-tocopherol, 2,2′-Azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS+), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4U/mg, Sigma), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), electric eel acetylcholinesterase (AChE, EC 3.1.1.7, 426U/mg, Sigma), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 426U/mg, Sigma), acetylthiocholine iodide, Neocuproine, butyrylthiocholine chloride and galantamine were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany).

Plant material
Biscutella raphanifolia (Brassicaceae) is an endemic species (Quezel et al., 1963), collected in June 2010, locally at Djebel El Ouahch (800 m altitude), Constantine (Eastern Algerian) and identified at Badji-Mokhtar university (Annaba) by Pr. Gérard De Bélair.

Extraction procedures
150g of the sprayed plant are macerated in a water-alcohol mixture (water/methanol; 80/20, v/v) for 24 hours at ambient temperature. The recovered extract was concentrated under reduced pressure at a moderate temperature (45°C). The method was repeated 3 times with replacement of the solvent in each case and lasts 24 to 48 hours. The three recovered alcoholic extracts were combined and concentrated. To the concentrated solution, 300ml of water were added. After filtration, a clear aqueous solution was obtained. This aqueous phase was 300ml of water were added. After filtration, a clear aqueous solution was obtained. This aqueous phase was combined, 20 main fractions were obtained. Fraction F-2 was separated on column of silica gel, using an isocratic elution system of AcOEt/MeOH/H2O (8/1/0.5:7/1/0.5) and TLC on polyamide DC6, eluted with H2O/MeOH/methyllethylketone/acetylecetone (13/3/3/1), affording four pure flavonoids which were identified by the use of spectral methods: UV, 1H NMR and 13C NMR and high multidimensional NMR experiment techniques.

Acid Hydrolysis
The acid hydrolysis of the pure products was achieved by the use of authentic samples of sugars (Lakhal et al., 2011).

Biological activities
Antibacterial activity
The study of the antibacterial activity (Bauer et al., 1966; NCCLS, 1993) of the PEBR, DEBR and BEBR was made against 5 pathovars of Escherichia coli: Enterotoxogenic E. coli (ETEC) ATCC 1493, Enteropathogenic E. coli (EPEC) ATCC 2348, Enterohemorrhagic E. coli (EHEC) ATCC HB 101, Enteroinvasive E. coli (EIEC) ATCC LT 10407, and Enteraggregative E. coli (EAEC) and Staphylococcus aureus ATCC 25923 (Gram-positive cocci), Pseudomonas aeruginosa ATCC 27853 (Gram-negative bacille) and Enterococcus feacalis ATCC 29212 (Gram-positive cocci).

DPPH free radical scavenging test
The free radical-scavenging activity has been performed according to Blois (Blois, 1958).

ABTS cation radical decolorization test
The ABTS+ scavenging activity has been tested according to Re (Re et al., 1999).

Cupric reducing antioxidant capacity (CUPRAC)
The results of this method were compared from the absorbance values with the standards: BHA and α-tocopherol (Apak et al., 2004).

Metal chelating activity
The metal chelating activity by the ferrren-Fe2+ complexation assay has been carried on following the method of Decker (Decker et al., 1990), the standard used in this method is ethylenediamine tetra acetic acid (EDTA).

Determination of acetylcholinesterase- (AChE) and butryrylcholinesterase- (BChE)
AChE and BChE inhibitory activities were determined by Ellman (Ellman et al., 1961). The results were compared with galantamine.

STATISTICAL ANALYSIS
The tests were performed in triplicate for the antioxidant and anti-cholinesterase activities, recorded as mean ± standard deviation (Anova). Student’s-t test procedures were used for determination of significant differences between means, p. values<0.05 were considered as significant.
RESULTS

Identification of isolated compounds

The phytochemical study of *Biscutella raphanifolia*, after successive splitoffs on different chromatographic supports, led to the isolation and identification of four flavonoids:

- Quercetin-3-O-β-D-glucoside (1) - 
  C_{21}H_{20}O_{12}, UV (MeOH, λ_{max}, nm): 269, 354; +NaOH: 267, 324, 402; +AlCl₃: 272, 407; +AlCl₃/HCl: 274, 377. 1H NMR (400 MHz, DMSO-d₆, δ, ppm, J/Hz): 7.74 (1H, d, J=2, H-2'), 7.50 (1H, dd, J=8.4, 2.0, H-6'), 6.77 (1H, d, J=8.4, H-5'), 6.30 (1H, d, J=2.0, H-8), 6.10 (1H, d, J=2.0, H-6), 5.06 (IH, d, J=7.6, H-1"Glc), 3-4 (sugar protons). 13C NMR (125 MHz, DMSO-d₆, δ, ppm) 178.9 (C-4), 165.1 (C-7),

**Table 1**: Antibacterial activity of the PEBR, DEBR and BEBR of *Biscutella raphanifolia*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition zone, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEBR</td>
</tr>
<tr>
<td>Enterohemorrhagic E. coli</td>
<td>13.5</td>
</tr>
<tr>
<td>Enterotoxigenic E. coli</td>
<td>17.5</td>
</tr>
<tr>
<td>Enteroinvasive E. coli</td>
<td>12.0</td>
</tr>
<tr>
<td>Enteroaggregative E. coli</td>
<td>15.5</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6.0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>13.0</td>
</tr>
<tr>
<td>Enterococcus fecalis</td>
<td>15.5</td>
</tr>
</tbody>
</table>

**Table 2**: The inhibition (%) of ABTS and the free radical scavenging activity by DPPH assay of extracts of *Biscutella raphanifolia*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>ABTS</th>
<th>DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.5µg</td>
<td>25.0µg</td>
</tr>
<tr>
<td></td>
<td>58.6±1.67</td>
<td>82.9±1.79</td>
</tr>
<tr>
<td></td>
<td>69.71±0.94</td>
<td>83.61±0.42</td>
</tr>
<tr>
<td></td>
<td>59.54±1.04</td>
<td>77.93±0.61</td>
</tr>
<tr>
<td></td>
<td>14.07±1.09</td>
<td>22.48±0.20</td>
</tr>
</tbody>
</table>

Values expressed are means ± S.E.M. of three parallel measurements (p<0.05).

**Table 3**: The cupric reducing antioxidant capacity (CUPRAC) by Cu²⁺ Cu⁺ transformation and metal chelating activity (Inhibition %) by Ferrene-Fe²⁺ assays of the extracts of *Biscutella raphanifolia*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>% Inhibition</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metal chelating</td>
<td>CUPRAC</td>
</tr>
<tr>
<td></td>
<td>12.5µg</td>
<td>25.0µg</td>
</tr>
<tr>
<td>BEBR</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>EEBR</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>DEBR</td>
<td>1.49±0.23</td>
<td>4.69±1.22</td>
</tr>
<tr>
<td>PEBR</td>
<td>2.41±1.44</td>
<td>5.45±1.66</td>
</tr>
</tbody>
</table>

Values expressed are means ± S.E.M. of three parallel measurements (p<0.05). na: Not active

**Table 4**: Acetyl- and butyryl-cholinesterase inhibitory activities of extracts of *Biscutella raphanifolia*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>AChE</th>
<th>BChE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25µg</td>
<td>50µg</td>
</tr>
<tr>
<td>BEBR</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>EEBR</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>DEBR</td>
<td>2.40±0.78</td>
<td>3.14±1.79</td>
</tr>
<tr>
<td>PEBR</td>
<td>68.4±1.1</td>
<td>74.4±0.7</td>
</tr>
</tbody>
</table>

aValues expressed are means ± S.E.M. of three parallel measurements (p<0.05). bReference compounds. na Not active.
161.8 (C-5), 158.5 (C-9), 156.2 (C-4'), 149.1 (C-2), 145.3 (C-3'), 136.4 (C-3), 123.1 (C-6'), 122.7 (C-1'''), 116.3 (C-5'), 116.2 (C-2'), 114.1 (C-10), 101.2 (C-1'''), 98.5 (C-6), 94.6 (C-8), 78.1 (C-3''), 76.3 (C-5''), 75.0 (C-2''), 70.4 (C-4''), 61.9 (C-6''). Acid hydrolysis of this compound produced quercetin and D-glucose. Spectral data were compared with previously published data (Touafek et al., 2011).

**Quercetin-3-O-β-D-glucosyl(1→2)-O-β-D-glucoside [2] - C_{37}H_{30}O_{17} (Mohamed et al., 2001).**

Quercetin-3-O-β-D-glucosyl(1→3)-O-β-D-glucoside [3] - C_{38}H_{30}O_{18}, UV (MeOH, λ_{max}, nm): 271, 321, 365, +AlCl3: 274, 393, +AlCl3/HCl: 275, 424; NaOAc: 270, 368. +H$_3$BO$_3$: 264, 380. $^1$H NMR (400 MHz, MeOD, δ, ppm, J/Hz): 7.72 (1H, d, J=2.2, H$_2''''$), 7.6 (1H, d, J=10.6-2.2, H6''), 6.89 (1H, d, J=8.5, H5''), 6.41 (1H, d, J=2.1, H-8), 6.22 (1H, d, J=2.2, H-6), 5.32 (1H, d, J=7.7, H-1'''' glucose), 4.62 (1H, d, J=7.8, H-1''''' glucose), 3.20-4.00 (sugar protons). 13C NMR (400 MHZ, MeOD, δ) 177.9 (C-4), 165.2 (C-7), 161.6 (C-5), 157.4 (C-9), 157.1 (C-1''), 148.5 (C-2'), 144.5 (C-3''), 134.1 (C-3), 121.8 (C-1''), 121.6 (C-6''), 116.1 (C-2'', C-5''), 114.6 (C-10), 104.1 (C-6), 103.8 (C-8), 100- 60 (sugar carbons). HMBC experiment established a correlation between C-3 and H-1'', C-2'' and H-1'''', coherence (HSQC) experiments, the HMBC experiment which correlation between C-3 and H-1'', C-2'' and H-1'''', C-9''' and H-6'''' were observed (Wang et al., 2010).

**Kaempferol-3-O-β-D-glucosyl(1→2)-6''''p-coumaryloxy-β-D-glucoside [4] - C_{41}H_{32}O_{18}, UV (MeOH, λ_{max}, nm): 269, 314; +NaOH: 276, 321, 365, +AlCl3: 276, 399; +AlCl3/HCl: 277, 399, NaOAc: 275, 374. +H$_3$BO$_3$: 275, 372. $^1$H NMR (400 MHz, MeOD, δ, ppm, J/Hz): 8.02 (2H, d, J=8.8, H-2', H-6'), 7.35 (2H, d, J=15.9, H-7''''), 7.27 (2H, d, J=8.5, H-2'''', H-6''''), 6.89 (2H, d, J=8.8, H-3', H-5'), 6.7 (2H, d, J=8.6, H-3''', H-5'''), 6.32 (2H, d, J=2.0, H-8'), 6.16 (2H, d, J=2.0, H-6), 6.04 (1H, d, J=16.0, H-8'''''), 5.21 (1H, d, J=5.2, H-1'''' glucose), 4.66 (1H, d, J=7.6, H-1''''' glucose), 3.20-4.40 (sugar protons). Acid hydrolysis of 4 produced kaempferol and D-glucose. Assignment of glucosidic protons system was achieved by analysis 1H-1H COSY and heteronuclear single quantum coherence (HSQC) experiments, the HMBC experiment which correlation between C-3 and H-1'', C-2'' and H-1'''', C-9''' and H-6'''' were observed (Wang et al., 2010).

**Anti-bacterial activity**

Antibacterial tests were carried on EEBR, DEBR and BEBR against several strains (reference strains and isolated from pathological germs samples). As reported in table 1.

**Anti-oxidant activity**

In their radicalic forms, DPPH free and ABTS cation radicals absorb at 517 nm and 734 nm, respectively. DPPH$^-$ and ABTS$^-$ scavenging activities of the extracts are shown in table 2.

Table 3 shows: the cupric reducing antioxidant capacity when the absorbance in this method is measured at 450nm, the results obtained are compared with the standards BHA and α-tocopherol at 0.1mg/ml (Absorbance 3.51 ± 0.01 and 1.85 ± 0.01 respectively) and the chelating effects were compared with standard on ferrous ions EDTA at 0.1 mg/ml (92.5±1.4%).

**Anti-cholinesterase activity**

Table 4 shows AChE and BChE inhibitory activities of BEBR, EEBR, DEBR and PEBR, compared with that of the standard (galantamine), used to treat mild Alzheimer’s disease. The tests were carried on spectrophotometrically in a 96 well plate microplate reader at 25, 50, 100 and 200 µg/mL concentrations.

**DISCUSSION**

**Anti-bacterial activity**

The PEBR exhibited the best antibacterial activity against ETEC (17.5mm), EPEC (15.5mm) and Enterococcus fecalis (15.5 mm), whereas the EHEC strain was more sensitive to the DEBR (14.5) and BEBR (15 mm) with respective inhibition zone diameters.

**Anti-oxidant activity**

For this study, we hypothesized that plants are a potential source of natural antioxidants. The results observed in antioxidant tests have confirmed this hypothesis.

The absorbance decreases by the reduction effect of antioxidants. Because of the steric hinderance, ABTS$^-$ assay has a superior effect than the DPPH$^-$ one.

The BEBR (IC$_{50}$=18.80±0.33 µg/ml), and EEBR (IC$_{50}$=15.76±0.83 µg/ml) showed better activities compared to the reference BHA (IC$_{50}$=45.37±0.47 µg/ml), and close to the reference α-tocopherol (IC$_{50}$=7.31±0.17 µg/ml). In the ABTS assay, however, the BEBR (IC$_{50}$=10.24±0.32 µg/ml) demonstrated good activities compared to references BHA (IC$_{50}$=4.10±0.06 µg/ml) and α-tocopherol (IC$_{50}$=4.31±0.10 µg/ml).

In the metal chelating method, the EEBR did not exhibit antioxidant activity but the BEBR and DEBR have a very small percentage of inhibition. The PEBR showed the best antioxidant activity in this method at 0.1 mg/ml (32.78±1.18%). However, none of the latter antioxidant effects were close to that of EDTA.
In the cupric reducing activity, the BEBR showed a better or close activity to the reference α-tocophérol.

**Anti-cholinesterase activity**

The BEBR and EEBR were unable to indicate anti-cholinesterase activity. The DEBR and EEBR, however showed low activity against acetylcholinesterase and butyrylcholinesterase.

**CONCLUSION**

Secondary metabolites and biological effects of *Biscutella raphanifolia* are reported here for the first time. The phytochemical study of the aerial parts of this endemic species has led to the separation and identification of four flavonol glycosides. The PEBR exhibited the best species has led to the separation and identification of four phytochemical study of the aerial parts of this endemic. The four studied extracts showed excellent antioxidant and antibacterial activities. However, the PEBR and DEBR showed a very weak anti-cholinesterase activity.

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**REFERENCES**


