Full Length Article

LC/MS characterization of antioxidant flavonoids from Tragia involucrata L.

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ABSTRACT

Chromatographic and spectroscopic methods were developed for the identification of flavonoids with potent radical scavenging activity from Tragia involucrata, an important medicinal plant. The separation consisted of column chromatography and high pressure preparative liquid chromatography. In vitro radical scavenging activity of different fractions was screened by DPPH radical scavenging assay. The ESI MS/MS analysis was employed for the characterization of active fractions. Flavonoids such as iridin, dihexosyl quercetin, quercetin-3-O-rutinoside, rhamnosyl hexosyl methyl quercetin, gentenstein 7-glucoside, orientin, C-(O-caffeoyl-hexosyl)-O-hexoside and tricin 7-O-hexosyl-O-hexoside were identified, for the first time, from T. involucrata.

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1. Introduction

Tragia involucrata has been widely used in traditional systems of medicine for a variety of diseases (Samy et al., 2006). The root has been reported to possess diaphoretic, wound healing, antimicrobial and alterative properties (Gobalakrishnan et al., 2013; Samy et al., 2006) and was used for treating many diseases such as asthma, fevers, diarrhea, excessive urination, vomiting, dermatoses, cold, leg and arm pain, skin eruptions, venereal diseases and fever (Ajithkar et al., 1999; Ayyanar and Ignacimuthu, 2005; Chopra, 1958).

Many properties of plant products are associated with the presence of phenolic compounds, which are essential for plant development and play an important role in their defense mechanisms. The plant phenolics and flavonoids have powerful biological activity, which outline the necessity of their characterization (Sulaiman et al., 2014a). Phenolics are the most abundant secondary metabolites of plants, with more than 8000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins (Dai and Mumper, 2010).

Flavonoids have been considered powerful free radical scavengers and proved to be more potent antioxidants than vitamins and carotenoids (Dai and Mumper, 2010). Polyphenolics are among the most desirable food bioactive compounds because of their significant antioxidant activity (Naczk and Shahidi, 2006; Rice-Evans et al., 1996). The objective of the present investigation was to separate and characterize flavonoids from T. involucrata.

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2. Materials and methods

2.1. Plant material

The whole plant of T. involucrata (700 g) was collected from the Herb garden of Arya Vaidya Sala, Kottakkal, India, during July 2011 and was authenticated by the Plant Systematics and Genetetic Resources Division of the Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala, Kottakkal, Kerala, India, and voucher specimen was deposited in the ‘CMPR’ herbarium.

2.2. Instruments and chemicals

DPPH radical scavenging assay was done using a UV–visible spectrophotometer (Pharmaspec-1700, Shimadzu, Japan). Separation of various fractions was carried out on silica gel (Merck, 230–400 Mesh). TLC was done on a precoated silica plate (F254, Merck). HPLC analysis was done by Agilent 1200 Preparative High Pressure Liquid Chromatographic system equipped with prep pump, a Rheodyne injector, Diode Array Detector in combination with Chem32, Chemstation software. LC-ESI-MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with Extend-C18 column of 1.8 μm, 2.1 × 50 mm. Folin–Ciocalteu reagent was procured from Sisco Research Laboratory (SRL), Mumbai, India. DPPH, catechin, gallic acid and quercetin were procured from Sigma Chemicals Co. (Bangalore, India). All other chemicals employed were of standard analytical grade from Merck India.

2.3. Extraction

The shade dried materials were extracted with 80% aqueous methanol using Soxhlet method for 72 hours. The final extract was concentrated to dryness on a rotary evaporator at 40 °C. It was then dissolved in 250 ml of HPLC grade methanol.

2.4. Separation of flavonoids

The aqueous alcoholic extract was partitioned into chloroform and butanol. The preliminary chemical analysis of butanol and chloroform fractions was done using thin layer chromatography on a pre-coated silica gel (60F254, Merck India) using toluene and ethyl acetate in the ratio of 8:2 as mobile phase. The chromatogram was developed in a saturated chromatographic chamber (Camag, Switzerland) and evaluated under UV at 254 nm. BuOH fraction was separated on a silica gel (230–400 mesh) column (90 × 5 cm) eluting with a gradient solvent system (CHCl3—MeOH) to give 10 main fractions (Fr A1–A10). Fractions Fr A5–A8 were clubbed together on the basis of TLC pattern and were further separated over silica gel column eluted with EtOAc—MeOH—H2O (80:10:10 to 20:40:40) to yield six subfractions (Fr B1–B6). Fr B6 was yet again chromatographed on silica eluted with 100% methanol and yielded an amorphous pale yellow powder. It was subsequently purified with preparative HPLC by peak based fraction collection (Compound 1). The characterization of this compound is on progress which will be reported later. The remaining fractions (Fr B1–B5) were stored in separate vials until LC/MS characterization.

2.5. DPPH radical scavenging activity

Different subfractions (Fr B1–B5) were screened for their radical scavenging activity using DPPH, which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was added to the solutions prepared with various fractions and standard catechin and was stirred. Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank (Shimada et al., 1992).

2.6. LC/MS characterization of active fractions

LC-ESI-MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with Extend-C18 column of 1.8 μm, 2.1 × 50 mm. Gradient elution was performed with water/0.05% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.2 ml/min. MS parameters for mass fragmentation were optimized by previous methods (Sulaiman et al., 2014b).

2.7. Statistical analysis

Data were presented as mean ± standard deviation (SD) of three determinations. Results were calculated by employing statistical software (COSTAT, Monterey, CA, USA).

3. Results and discussions

3.1. Preliminary characterization and separation of Compounds

The aqueous alcoholic extract was partitioned into chloroform and butanol fractions. Preliminary analysis was done using TLC. On evaluation at 254 nm, butanol fraction showed major bands at R: 0.10, 0.31, 0.40, 0.54, 0.61, 0.73 and 0.81, while major bands were observed at 0.14, 0.19, 0.32 and 0.72 for chloroform fraction (Fig. 1). Butanol fraction was further separated on silica gel to yield ten fractions. On the basis of TLC analysis,
fractions 5A, 6A, 7A and 8A were clubbed. The pooled fraction was separated again on silica gel and yielded six subfractions (Fr B1, Fr B2, Fr B3, Fr B4, Fr B5 and Fr B6). Fractions FrB1–B5 were screened for radical scavenging activity using DPPH. Fr B6 was subjected to further separation and purification and yielded a pale white powder. The characterization of the same will be reported later.

### 3.2. DPPH radical scavenging activity

DPPH radical is one of the free radicals widely used for testing the preliminary radical scavenging activity of the plant extract (Deshpande et al., 2013) as it is a direct and reliable method for determining radical scavenging activity (Aksoy et al., 2013). The subfractions Fr B1, Fr B2, Fr B3, Fr B4 and Fr B5 were screened for their radical scavenging activity in vitro using DPPH. Fractions Fr B3 and Fr B4 showed significant DPPH radical scavenging capacity with EC$_{50}$ of 4.58 and 4.26 respectively (Table 1). The DPPH radical scavenging activity of ethyl acetate extract of *T. involucrata* was reported by Joshi et al. (2011) with an EC$_{50}$ of 6.46 ± 0.371/µg/ml. In the present study, fraction B4 showed more activity toward the DPPH radicals.

### 3.3. LC/MS characterization of active fractions

Fractions Fr B3 and Fr B4 were found to be most active fractions against DPPH radicals. The characterization of the same was performed by LC-ESI-MS/MS analysis in positive mode. Mass fragmentation was performed on collision induced dissociation (CID) by varying the collision cell voltage. Fr B3 showed five peaks (Fig. 2, Table 2) on extracting the total ion chromatogram (TIC) into base peak chromatogram (BPC). At 0.82 minute, the peak corresponding to [M + H]+ 523.42 was identified as Iridin, an isoflavone, since the fragmentation pattern (m/z 361, 310 and 282) was in consistent with the previous reports (Bhat et al., 2014). Peaks at 1.03 1.65 and 2.07 were identified as quercetin derivatives such as dihexosyl quercetin, quercetin-3-O-rutinoside and rhamnolysyl hexosyl methyl quercetin respectively. The fragmentation of these compounds was reported earlier (Crupi et al., 2014; Felipe et al., 2014; Ding et al., 2006). The peak at m/z [M + H]+ 433.28 was identified as Gentenstein 7-glucoside as it presented major fragments at m/z 392, 352, 304 and 280, which were in agreement with earlier reports (Bhat et al., 2014).

Fraction Fr B4 yielded four major peaks on extracting the BPC (Fig. 3, Table 3). At 0.83, a peak with m/z 449.10 fragmented on collision induced dissociation (CID) with a major fragment of m/z 286, which was consistent with the fragmentation pattern of orientin (de Oliveira et al., 2013; Ma et al., 1997). Peak at 2.47 (m/z [M + H]+ 773.18) was identified as caffeoylhexosyl-O-hexoside as it presented major fragments with m/z 449 and 431. It was in agreement with the previously reported fragmentation pattern of the caffeoyl derivative (Ma et al., 1997; Wojakowska et al., 2013). The molecular ion at 3.12 showed MS spectrum with m/z 655.18 and it was fragmented as Tricin 7-O-hexosyl-O-hexoside on the basis of mass fragmentation of

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**Table 1 – DPPH radical scavenging activity of various fractions of *T. involucrata.***

<table>
<thead>
<tr>
<th>Fraction/standard</th>
<th>EC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr B1</td>
<td>12.78 ± 0.18</td>
</tr>
<tr>
<td>Fr B2</td>
<td>9.76 ± 0.24</td>
</tr>
<tr>
<td>Fr B3</td>
<td>4.58 ± 0.26</td>
</tr>
<tr>
<td>Fr B4</td>
<td>4.26 ± 0.16</td>
</tr>
<tr>
<td>Fr B5</td>
<td>6.38 ± 0.24</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.40 ± 0.12</td>
</tr>
</tbody>
</table>

**Table 2 – LC-MS/MS analysis of Fr B3.**

<table>
<thead>
<tr>
<th>R$_i$</th>
<th>m/z [M + H]+ (experimental)</th>
<th>m/z [M + H]+ (calculated)</th>
<th>Error in ppm</th>
<th>MS/MS</th>
<th>Tentative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.82</td>
<td>523.4254</td>
<td>523.4634</td>
<td>0.038</td>
<td>361, 310, 282</td>
<td>Iridin</td>
</tr>
<tr>
<td>1.03</td>
<td>627.1480</td>
<td>627.158</td>
<td>0.001</td>
<td>303</td>
<td>Dihexosyl quercetin</td>
</tr>
<tr>
<td>1.65</td>
<td>610.1259</td>
<td>610.1541</td>
<td>0.0282</td>
<td>464, 302</td>
<td>Quercetin-3-O-rutinoside</td>
</tr>
<tr>
<td>2.07</td>
<td>625.0863</td>
<td>625.1808</td>
<td>0.0945</td>
<td>479, 317</td>
<td>Rhamnosyl hexosyl methyl quercetin</td>
</tr>
<tr>
<td>3.24</td>
<td>433.2840</td>
<td>433.1033</td>
<td>0.1807</td>
<td>392,352,304,280</td>
<td>Gentenstein 7-glucoside</td>
</tr>
</tbody>
</table>
previous reports (Cavaliere et al., 2005; Chen et al., 2013; Ma et al., 1997).

4. Conclusion

The chromatographic separation and spectroscopic studies of active fractions from T. involucrata led to the identification of eight flavonoids which were first reported from this species. The current studies also showed that T. involucrata is a natural source for flavonoids with potent free radical scavenging activity.

Acknowledgement

The financial assistance from Tata Trusts, Mumbai, is gratefully acknowledged.

REFERENCES


Table 3 – LC-MS/MS analysis of Fr B4.

<table>
<thead>
<tr>
<th>Rt</th>
<th>m/z [M + H]+ (experimental)</th>
<th>m/z [M + H]+ (calculated)</th>
<th>Error in ppm</th>
<th>MS/MS</th>
<th>Tentative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.83</td>
<td>449.1071</td>
<td>449.3863</td>
<td>0.2792</td>
<td>286</td>
<td>Orientin</td>
</tr>
<tr>
<td>2.47</td>
<td>773.1804</td>
<td>773.1906</td>
<td>0.0102</td>
<td>449, 431</td>
<td>C-[O-caffeoyl-hexosyl]-O-hexoside</td>
</tr>
<tr>
<td>3.12</td>
<td>655.1852</td>
<td>655.1015</td>
<td>0.0837</td>
<td>475, 371, 330</td>
<td>Tricin 7-O-hexosyl-O-hexoside</td>
</tr>
</tbody>
</table>


