Antioxidant and anti-inflammatory activity of phenolic extracts of Genista ferox (Fabaceae)

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Abstract: Phenolic extracts of aerial parts of Genista ferox have been characterized and evaluated for their pharmacological properties which are still not reported. The total phenol and flavonoid contents in the extracts were estimated spectrophotometrically via the Folin-Ciocalteu and aluminium chloride methods, respectively. Leaves and pods extracts showed the highest contents of total phenols and total flavonoids. The same extracts exhibited also the highest antioxidant capacity (IC50 of 105.37µg/mL and 113.98µg/mL, respectively) assessed by the in-vitro DPPH radical scavenging method. Leaf and stem extracts were explored for their possible anti-inflammatory activity assayed by carrageenan-induced paw edema model. Both extracts (at 400mg/kg) showed edema inhibitory effect, which was found to be close to that of Dichlofenac reference. However, the leave extract produced the highest significant (p<0.001) anti-inflammatory activity when compared with the control. A preliminary characterisation of these phenolic extracts were carried out by high performance liquid chromatography (HPLC) coupled with diode-array detector (DAD), in order to determine the relevance of identified compounds in the pharmacological properties of the plant.

Keywords: Antioxidant, anti-inflammatory, Genista ferox, phenolics, HPLC-DAD.

INTRODUCTION

Genista L. (Fabaceae) is a large genus of shrubs mainly centred in the Mediterranean region that is related to the European, North African and West Asian regions. In Algeria, the genus is represented by 25 species, among which 11 species are endemics (Quezel and Santa, 1963). Some of these plants have traditionally been used for the treatment of gastrointestinal disorders and to control hyperglycemia.

Previous studies on the phytoconstituents of the genus Genista reported the presence of various bioactive molecules, notably predominant flavonoids (Pistelli et al., 1998) which are potentially bioactive molecules (Harborne and Williams, 2000); alkaloids (Küçükboyacı et al., 2012); and recently saponins (Boutaghane et al., 2013). Many of Genista species have proved several biological properties, ulcerogenic (Rainova et al., 1988), hepatoprotective (Baali et al., 2014) anti-diabetic (Rauter et al. 2009) and anti-inflammatory capacity (Boubekri et al., 2014). This last have been, at least partially, attributed to antioxidant properties of their natural products, mainly phenolic compounds. Current researches have proved the effectiveness of phenolic extracts from different Genista species as antioxidant agents, using numerous tests (Orhan et al., 2011; Bouchouka et al., 2014; Baali et al., 2016; Hangaru et al., 2016).

In this paper, as a continuation of our research on algerian medicinal species with potential bioactivities (Boubekri et al., 2014; Chebbab et al., 2014; Ait-kaci Aourahoun et al., 2015; Baali et al., 2016), we report on an endemic Genista species, G. ferox, data on its phytochemistry and pharmacological activity are very scarce. In fact, only one study on its alkaloids (Faugeras and Rene, 1966) and flavonoids (Mekkiou, 2005) have been published to date. The objective of this current study was to characterize and evaluate antioxidant and anti-inflammatory activity of phenolic extracts of Genista ferox.

MATERIALS AND METHODS

Plant material and extraction procedure

Genista ferox samples were collected locally from Boumerdes (NE of Algeria) in April 2012. Authentication of the species was made by Dr. Errol Véla from Montpelier University (France). The whole plant was air dried at room temperature in shade and the different parts (leaves, stems, flowers and pods including seeds) were separately isolated and reduced to fine powder using a laboratory blender. The powdered materials (10g of each leaves and stems 5g of flowers and 2g of pods) were separately sonicated for 1h with 2 cycles in hour, in 80ml, 50ml, 100 ml and 20ml EtOH-H2O (80:20), respectively.
Ultrasonic extraction was conducted on Bandelin ultrasonic bath (35 kHz, 150 W). The alcohol was completely removed by rotary evaporator and further removal of water was performed by freeze-dry procedure.

**Estimation of total phenolics content**
The total phenolics content was determined by Folii-Cicalteu’s assay (Luis et al., 2009). Gallic acid was used as standard compound. Phenolic contents of samples are expressed in mg as Gallic acid equivalent (GAE/g of dry extract) based on the calibration curve (y = 0.015x - 0.0323; R² =0.9996).

**Determination of total flavonoids content**
The total flavonoids content was estimated according to Kiendrebeogo et al. (2005), using standard Quercetine for the calibration curve (y = 0.024x - 0.015; R² =0.997). Flavonoid contents were expressed as mg Quercetine equivalent (QE/g dry extract).

**Antioxidant activity**
**Thin layer chromatography-DPPH scavenging assay**
The screening of antioxidant potential of all hydroethanolic extracts was assayed according to Merian et al. (2014), with slight modifications. The extracts were chromatographed using TLC silica gel 60 F254 plates (10 x 10), using system solvent Ethyl acetate-Methanol-Water (100:13, 5:10, v/v/v). The DPPH test was performed directly on plates by spraying a 0.008% methanolic solution of DPPH to reveal the yellow zones with antioxidant activities.

**DPPH scavenging assay**
The antioxidant activity of leave, stem, flower and fruit extracts and standard ascorbic acid was evaluated by the radical scavenging assay method using DPPH (2,2-diphenyl-1-picyrylhydrazyl) free radical (Thakral et al., 2010). Radical scavenging activity is determined as the inhibition percentage (I%) computed as follows:

\[ I\% = \left( \frac{A_c - A_t}{A_c} \right) \times 100 \]

Where \( A_c \) is the absorbance of the blank sample and \( A_t \) is the absorbance of the tested sample.

Inhibition percentages were plotted against sample concentrations and IC₅₀ value (concentration of extract or standard required for 50% inhibition) were determined. All measurements were performed in triplicate.

**HPLC-DAD analysis**
The HPLC analysis was done on Agilent HPLC system model 1100 equipped with four pumps and Shimadzu SPD-20 AV-UV/Vis detector. 10µl samples of each extract previously dissolved in MeOH was injected. Reversed phase Hypersil BDS C₁₈ column (250 mm x 4.6 mm, 5µm) was used for chromatographic separation performed at room temperature. The elution phase consisted of acetonitrile (Solvent A) and 2% acetic acid in water (Solvent B), with gradient elution as described by Noumi, et al. (2011): 15% A/85% B; 0 to 12 min, 40% A/60% B 12 to 14 min, 60% A/40% B 14 to 18 min, 80% A/20% B 18 to 20 min, 90% A/10% B 20 to 24 min, 100% A 24 to 28 min. The flow rate of the mobile phase was kept at 0.5ml/min. Diode-array UV detection was performed at 280 nm for the monophenolic Gallic acid and 365 nm for the flavonoid Quercetin.

**Identification and quantification of phenolic compounds**
Phenolic Gallic acid and flavonoid Quercetine were initially identified by direct comparison of their retention times with those of standards. For the quantitative analysis, calibration curves were obtained by plotting the peak areas versus the tested concentrations range (0.25-100µg/mL) of each standard: Gallic acid (y = 47.8394x - 2.9396; R² =0.99968); Quercetine (y = 41.2466x - 7.5414; R² =0.99868). The results were expressed in µg per g of dry extract.

**In vivo Anti-Inflammatory activity**
**Animals**
Adult male mice *Mus musculus*, weighting 20-25g, were randomly divided into 6 experimental groups consisted of 6 mice each. The animals were housed under standard conditions, fed with Amrut brand pelleted standard diet with water *ad libitum*. The animals were deprived of food and water overnight to ensure uniform hydration. Institutional permission was obtained from Pharmacotoxicology Laboratory Head (Research and Development Center, Algiers), and approval guide (MO.C.LP.002) for the care and the use of laboratory animals was followed.

**Carrageenan-induced paw edema**
The screening of anti-inflammatory activity of leave and stem extracts was assayed by carrageenan-induced mouse paw edema according to Levy (1969). The extracts LE and SE diluted in sterile water and reference drug Diclofenac were orally administered (0.5ml) in doses 200mg/kg, 400mg/kg and 75mg/kg, respectively. The control animals received 0.5ml sterile distilled water. Following 30 min latency phase, edema was induced by subplantar injection of 25µl of carrageenan-saline solution (1% w/v) into the left hind paws of animals. Right paws served as the control (non-inflammed paws). The animals were sacrificed 4 hours after carrageenan injection and the right and the left paws were incised with a scalped at the tibiotarsal articulation for weighting, using an analytical balance. The edema percentage (E %) was calculated by the following equation:

\[ E \% = \left( \frac{L - R}{L} \right) \times 100 \]

Where \( L \) is the weight of the left paw and \( R \) is the weight of the right one of each mouse.

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The anti-inflammatory activity was expressed as percentage of the edema inhibition (EI) in treated mice compared to the control mice, using the formula:

$$EI \% = \left(\frac{C - T}{C}\right) \times 100$$

Where C is edema percentage of the control group and T is edema percentage test group.

### STATISTICAL ANALYSIS

Statistical analysis was performed using Student’s t-test to determine significant differences between the control and the treated groups, using SPSS (version 9) statistical software. The data are represented as mean ± SEM. Significant differences were considered at $p \leq 0.05$.

### RESULTS

**Determination of total phenols and flavonoids**

The aerial parts of *G. ferox* were extracted by ultrasound assisted extraction process. The average percentage yields of hydro-ethanolic extracts were found to be: 15.15% (stems), 23.15% (leaves), 24.75% (pods) and 32.2% (flowers).

The total phenolic contents (TPC) expressed as mg GAE/g dry extract and total flavonoid contents determined as mg QE/g dry extract are presented in table 1.

![Fig. 1: TLC chromatograms of leaf extract (LE), stem extract (SE), flower extract (FE) and pod extract (PE); before (A) and after (B) spraying of methanolic DPPH solution.](image)

The highest amounts of phenolics were determined for leaf extract LE (177mg GAE/g) and pod extract PE (165.33 mg GAE/g) which were also more richer in flavonoids (164.13mg QE/g and 120.05mg QE/g, respectively) than other samples, whereas the lowest contents in these compounds were recorded in flower extract FE.

**Screening and evaluation of DPPH scavenging activity of phenolic extracts**

Before DPPH scavenging assay, TLC-DPPH test was performed to screen our extracts for antioxidant compounds. Observation of the plates revealed several yellow bands before and after spraying of methanolic DPPH solution, with important yellow spot at Rf=0 observed notably in leaf extract (fig. 1). This indicates the contribution of flavonoids and other probably phenolic compounds in the reduction of DPPH radical.

All investigated extracts showed moderate to weak antioxidant activity than that of the positive standard ascorbic acid (IC$_{50}$ 16.16µg/ml). LE and PE were more potent than those FE and SE, with IC$_{50}$ 105.37µg/ml and 113.98µg/ml, respectively. The scavenging effect of the samples on the DPPH radical decreased by increasing of IC$_{50}$ values according to the following order: ascorbic acid > LE > PE > FE > SE.

**Qualitative and quantitative HPLC-DAD analysis**

Two phenolic compounds, flavonoid Quercetine and phenolic acid Gallic acid, were selected to allow justification of biological activities of leave and stem extracts. Quantification of Quercetin and Gallic acid, were made by measuring their absorbance in extracts at 365 nm for Quercetin and at 280 nm for Gallic acid. Both extracts showed very similar chromatographic fingerprint at both 280nm and 365nm. However, identified compounds were in largely different concentrations (tab. 2). Gallic acid was 2 fold higher in SE than in leave one (355.45µg/g extract versus 167.5µg/g extract), whereas Quercetine content in LE was 7 fold higher than in SE (14.60 µg/g extract versus 2.34µg/g extract).

**Anti-inflammatory activity**

The effects of leave and stem extracts from *G. ferox* on carrageenan-induced mice paw edema, are summarized in table 3. Four hours after intraplantar injection of carrageenan, both stem and leave extracts of *G. ferox* elicited a dose dependent edema inhibition. The extracts were found to exhibit maximum reduction in paw weight at the dose 400 mg/Kg (SE 13.22%, p<0.01 and LE 11.36%, p<0.001). The most active extract was that of LE, with edema inhibition value of 66.42%, while SE presented EI value that was close to Diclofenac reference (60.92% and 58.41% respectively).

### DISCUSSION

Phenolic compounds are mostly found in plants, and they have been reported to have a broad range of bioactivities, including antioxidant and anti-inflammatory properties.

The few reports on phenolic and flavonoid contents of *Genista* species revealed lower TPC (33.52mg GAE/g) and TFC (16.06mg QE/g) of aqueous ethanolic extract (70%) from aerial parts of *G. tinctoria* (Hanganu et al., 2016). However, higher TPC and TFC were obtained from aerial parts of *G. vuralii* (212.24mg GAE/g and 203.82 mg QE/g) and *G. sandrasica* (166.94 mg GAE/g and 203.82 mg QE/g).
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Table 1: Total phenols (mg/g extract as gallic acid equivalent), total flavonoids (mg/g as quercetin equivalent) and scavenging activity against DPPH radical (IC$_{50}$ value) of G. ferox extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>TPC (mg GAE/g)</th>
<th>TFC (mg QE/g)</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE</td>
<td>177 ± 2</td>
<td>164.13 ± 3.15</td>
<td>105.37 ± 0.6</td>
</tr>
<tr>
<td>SE</td>
<td>107.66 ± 3.92</td>
<td>61.52 ± 1.4</td>
<td>206.61 ± 2.92</td>
</tr>
<tr>
<td>FE</td>
<td>73.66 ± 0.88</td>
<td>29.52 ± 0.83</td>
<td>174.47 ± 1.16</td>
</tr>
<tr>
<td>PE</td>
<td>165.33 ± 3.17</td>
<td>120.05 ± 1.65</td>
<td>113.98 ± 1.72</td>
</tr>
</tbody>
</table>

TPC: Total phenolic content; TFC: total flavonoid content; LE: leave extract; SE: stem extract; FE: flower extract; PE: pod extract. Results are Means ± SEM (n=3).

Table 2: Concentration of Phenolic compounds (µg/g extract) in stem and leave extracts of G. ferox.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt (min)</th>
<th>LE (µg/g)</th>
<th>SE (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>6.576</td>
<td>167.5</td>
<td>355.45</td>
</tr>
<tr>
<td>Quercetin</td>
<td>23.204</td>
<td>14.60</td>
<td>2.34</td>
</tr>
</tbody>
</table>

Rt: retention time; LE: leave extract; SE: stem extract.

Table 3: Anti-inflammatory effect of G. ferox extracts on carrageenan-induced pawedema in mice.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Dose (mg/kg)</th>
<th>E %*</th>
<th>EI %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (water)</td>
<td>-</td>
<td>33.83 ± 2.23</td>
<td>-</td>
</tr>
<tr>
<td>SE</td>
<td>200</td>
<td>20.76 ± 5.3*</td>
<td>38.63</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>13.22 ± 4.58**</td>
<td>60.92</td>
</tr>
<tr>
<td>LE</td>
<td>200</td>
<td>27.82 ± 2.78</td>
<td>17.76</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>11.36 ± 1.66***</td>
<td>66.42</td>
</tr>
<tr>
<td>Dichlofenac</td>
<td>75</td>
<td>14.07 ± 2.76**</td>
<td>58.41</td>
</tr>
</tbody>
</table>

E: edema; EI: edema inhibition. *Values are Mean ± SE (n = 6); *p<0.05, **p<0.01, *** p<0.001 compared to control.

Fig. 2: HPLC-DAD chromatograms of standards Quercetin (A) at 365 nm and Gallic acid (B) at 280 nm.

and 158.06mg QE/g), extracted with methanol under ultrasonic vibrations (Orhan et al., 2011).

The scavenging DPPH radical assay is widely used to evaluate the free radical scavenging ability of natural antioxidant products. The concentration of antioxidant required to decrease the initial DPPH concentration by 50% (IC$_{50}$) is a parameter that allows the determination of the antioxidant effectiveness, which corresponds with a lower IC$_{50}$ value. Comparing to other species of genus Genista, phenolic extracts of G. ferox exhibited a higher level of radical scavenging than the hydro-ethanolic extract from G. sagittalis with IC$_{50}$ 304.05 µg/ml (Hanganu et al., 2016) and methanolic extracts of G. vuralii and G. sandrasica which expressed IC$_{50}$ values of 0.98 mg/mL and 1.11mg/mL, respectively (Orhan et al., 2011). However, hydro-ethanolic extract from aerial parts of G. tinctoria was shown to cause the best scavenging activity with IC$_{50}$ 65.45µg/ml (Hanganu et al., 2016).

On another hand, it is interesting to note that despite its high contents in total phenolics and flavonoids, the stem extract showed the weakest DPPH scavenging ability (IC$_{50}$ 206.61µg/ml). Similar results were found by Brighente et al. (2007); Bouchouka et al. (2012) and Chebbah et al. (2014). Therefore, considering all tested extracts of G. ferox, a lower correlation coefficients were found between TPC and TFC and IC$_{50}$ ($R^2$=0.71 and $R^2$=0.73, respectively). However, excluding the stem extract, a high linear correlations were established between TPC and TFC and DPPH scavenging activity ($R^2$=0.999 and $R^2$=0.955, respectively). This could be related to the different contents of these extracts in efficient DPPH scavenger compounds, such as identified...
Gallic acid and Quercetin. In fact, the effect of these compounds on DPPH radical scavenging has already been demonstrated. Previous studies reported the values of IC\textsubscript{50} 2.1 µg/mL (Brighente et al., 2007) and IC\textsubscript{50} 1µg/mL (Lu et al., 2002) for Quercetin. For Galliq acid, the values of IC\textsubscript{50} 2.6µg/mL (Brighente et al., 2007) and 1.5µg/ml (Lu et al., 2014) have been established. This clearly indicates that antioxidant activity of Quercetine, more concentrated in the LE, is slightly greater than that of Gallic acid.

The capacity of anti-inflammatory agents to inhibit mediators of inflammation is the basis of carrageenan-induced inflammation assay. These mediators include serotonin, histamine and bradykinin, which are responsible for the induction of inflammation in the first phase. The second late phase of inflammation is mediated by cyclooxygenase products, prostaglandins and lipoxygenase products prostaglandins that are released from the 3rd-4th hour (Vinegar et al., 1969). According to Winter et al. (1962), during the second phase of carrageenan-induced edema, compounds which are anti-inflammatory agents are detected as a result of decrease of prostaglandin concentration. As shown in the table 3, there was a high significant percentage inhibition of paw edema, at 4th hour by hydroalcolic leave extract (66.42%, P<0.001) and stem extract (60.92%, P<0.01) at dose of 400 mg/kg. Therefore, it can be inferred that the extracts possibly act by inhibiting the synthesis and/or action of prostaglandin since the extracts showed a significant inhibitory activity at the late phase of the edema development.

In addition, the high contents in phenolics of leave and stem extracts could be, at least partially, responsible for their anti-inflammatory effect. In fact, the potential of these compounds to scavenge free radicals and modulate inflammatory reactions has been demonstrated (Lee et al., 2007). Moreover, inhibition of cyclooxygenase and lipoxygenase has been attributed to many phenolics, such as flavonoids, in addition to their suggested ability to inhibit neutrophil degranulation which leads to diminution of the release of arachidonic acid by neutrophils and other immune cells (Robert et al. 2001).

The anti-inflammatory activities exhibited by both LE and SE could also be partially explained by the presence of identified compounds. Kim et al. (2005) provided that Gallic acid inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression. Quercetin have been found of to have significant anti-inflammatory activity (P<0.05) against carrageenan induced paw edema and cotton pellet granuloma mode in guinea pigs (Kaidama and Gacche, 2015). Another current report shown that Quercetin displayed inhibitory effects on prostaglandin E2 production in lipopolysaccharide (LPS)-stimulated macrophages (Seo et al., 2016). Furthermore, a recent study showed that the efficacy of Quercetin to inhibit cyclooxygenase enzymes COX-1 and COX-2 (measured by determined IC\textsubscript{50} for both enzymes) was mostly higher compared to that Gallic acid (4.9-69 and 10-3,01, respectively) (El-Hawary et al., 2016). That might also partly explain the high anti-inflammatory efficiency of leave extract compared to that stem one.
CONCLUSION
The present study reports the first data on the phenolic content and the pharmacological properties about *G. ferox*. Our study indicated that among all characterised phenolic extracts of aerial organs of *G. ferox*, those of leaves and pods showed highest contents of total phenols and total flavonoids. The same extracts exhibited also the highest antioxidant capacity. Evaluation of anti-inflammatory activity of leaves and stem extracts provided, at high tested dose, significant edema inhibitory effect, which was found to be close to that of Diclofenac reference. HPLC analysis indicated a higher content of leave extract in Quercetine while stem extract was found to be richer in Gallic acid.

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