Anti-inflammatory and antioxidant properties of Eriobotrya japonica leaves extracts

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Abstract
Background: In the present work we determined phenolic and flavonoids content of Eriobotrya japonica leaves extracts and fractions and their antioxidant and anti-inflammatory properties.

Objectives: To evaluate the inhibition of inflammatory PLA2 and antioxidant effects of extracts and fractions from Eriobotrya japonica leaves

Methods: Antioxidant activity was evaluated with DPPH radical scavenging assay and anti-inflammatory effect of fractions was measured by their inhibition potency on the human pro-inflammatory phospholipase A2 (group IIA).

Results: The EtOH/EtOAc 2:1 extract exhibited a potent inhibition of the hG-IIA with an IC50 values of 8 µg/ml. It also shows an antioxidant activity measured on DPPH with an IC50 of 42 µg/ml. Fractionation shows that CH2Cl2/MeOH 0:1 fraction was the rich one on flavonoids compounds (4.3 mg/g dry weight) and demonstrates a high antioxidant activity with an IC50 of 12 µg/ml. The anti-inflammatory evaluation demonstrates that the same fraction was the best one to inhibit the pro-inflammatory phospholipase A2 group IIA with an IC50 of 4 µg/ml.

Conclusion: Study conducted on Eriobotrya japonica shows that CH2Cl2/MeOH 0:1 fraction inhibits efficiently the hG-IIA phospholipase which is considered as pro-inflammatory enzyme.

Keywords: Eriobotrya japonica, extraction, flavonoids, anti-inflammatory.

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Introduction
Eriobotrya japonica Lindl, also known as ‘loquat’, belongs to the Rosaceae family. This plant is an evergreen shrub or small tree with narrow leaves that are dark green on the upper surface and have a lighter color under surface. It is originated from south-eastern China and later became naturalized in Korea, Japan, India and many other countries.

Leaves of Eriobotrya japonica (LEJ) Lindl (Rosaceae) have been used as traditional medicines for lung and stomach diseases and have been found to be effective in chronic bronchitis, inflammation, asthma, low back pain and tumor.1–3,4 Studies have demonstrated that LEJ has anti-inflammatory activity in a 12-O-tetradecanoylphorbol-13-acetate induced inflammation model. These reports strongly suggest that LEJ can be used as an anti-inflammatory agent.

Various triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides have been found in the LEJ and previous studies showed that some of these components have anti-tumor, antiviral, hypoglycemic, antioxidant and anti-inflammatory properties3,5–8.

During the inflammatory process, macrophages produce nitric oxide, cytokine and pro-inflammatory enzymes such as secreted phospholipase A2 (sPLA2)9,10 that catalyze the hydrolysis of membrane phospholipids to produce free arachidonic acid and lysophospholipids. Indeed, several studies showed that sPLA2 are the chief actors on the biosynthesis of lipid mediators in inflammatory cells11. sPLA2 enzymes are a heterogenic family that are divided on 11 groups (IB, IIA, IIC, IID, IIE, III, V, X, XIIA and XIIB)12–14. The sPLA2 group IIA was initially detected in synovial fluid of patients with rheumatoid arthritis15,16. Several studies demonstrated that the sPLA2 group IIA was involved
in inflammatory process and many phospholipases A2 inhibitors have been discovered and their effectiveness has been proved as a treatment of inflammatory disease.

Because overproduction of these inflammatory mediators might cause inflammatory damage, we focused in the present study on the evaluation of the anti-inflammatory effect of LEJ extracts by measuring the inhibition of the pro-inflammatory sPLA2 group IIA as well as their antioxidant activity.

Material and methods

Plant material

Leaves of Eriobotrya Japonica (Rosaceae) (LEJ) were collected in the region from Sfax (Tunisia) in June 2010. The plant was identified by Pr. M. Chaieb (Faculty of Sciences, Sfax University, Tunisia) and a voucher specimen has been deposited in the Chemical Laboratory of Natural Products (Sfax, Tunisia: No. LCSN 108).

Extraction and fractionation of flavonoids

The dry leaves of plant sample were ground to fine powder in a mill, and 100 g of powder was extracted in 1 L of MeOH/H2O 7:3. After filtration, the methanolic solution was evaporated and 250 mL of n-butanol was added. The organic phase was evaporated and the extract was dissolled in 200 mL of EtOH/0.5% BHT (2:1). The issue sample was separated on four fractions using CH2Cl2/MeOH at 8:2, 7:3, 5:5 and 0:1 proportion, respectively.

Total phenolic and flavonoids content

The issue sample was separated on four fractions using CH2Cl2/MeOH at 8:2, 7:3, 5:5 and 0:1 proportion, respectively. Briefly, 1.5 mL of DPPH solution at 10-5 M was incubated with 1.5 mL of extracts containing variable amounts of dry weight (between 0.01 and 1 mg). The reaction mixture was shaken and incubated in the dark for 30 min at room temperature. Control experiment was performed as described above without adding any LEJ extract. The OD of the solution was measured at 517 nm. The radical scavenging activity was calculated using the following equation:

\[
\text{Scavenging effect (\%)} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100
\]

The extract concentration provoking 50% inhibition (IC50) was calculated from the plot of the scavenging effect (percentage) against the extract concentration. BHT was used as standard.

DPPH radical scavenging assay

The antioxidant activity of LEJ extract and fractions were measured as equivalent of hydrogen-donating or radical scavenging ability, using the DPPH method with some modifications. Briefly, 1.5 mL of DPPH solution at 10-5 M was incubated with 1.5 mL of extracts containing variable amounts of dry weight (between 0.01 and 1 mg). The reaction mixture was shaken and incubated in the dark for 30 min at room temperature. Control experiment was performed as described above without adding any LEJ extract. The OD of the solution was measured at 517 nm. The radical scavenging activity was calculated using the following equation:

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Anti-inflammatory activity

The anti-inflammatory activity of extracts was followed by the inhibition of the human inflammatory phospholipase A2 group IIA (hG-IIA). The hG-IIA activity was measured as described by. Briefly, the substrate consisted of 3.5 mL methionin (Sigma Aldrich) in a mixture of 3 mM NaTDC, 100 mM NaCl, 10 mM CaCl2 and 0.055 mM red phenol as colorimetric indicator in 100 mL H2O. The pH of the reaction mixture was adjusted to 7.6. The hG-IIA or the pig pancreatic phospholipase A2 group IB (pG-IB) phospholipases were solubilized in 10% acetonitrile at a concentration of 0.02 and 0.002 µg/µL, respectively. A volume of 10 µL of these PLA2 solutions was incubated for 20 min at room temperature with 10 µL of each LEJ extracts and fractions. Then, 1 mL of the PLA2 substrate was injected in the medium, and the kinetic of hydrolysis was followed during 5 min by reading the decrease of OD at 558 nm. The inhibition percentage was calculated by comparison with a control experiment and the IC50 values were determined from the plot. The control experiment contained 10 µL of the enzyme (hG-IIA or pG-IB) and 10 µL of the corresponding organic solvent.

Statistical analysis study

Experimental results were given as mean value ± SD of three separate experiments. Statistical analysis was conducted using Microsoft Excel software using the Dunnett test performed after analysis of variance (ANOVA).

Table 1. Extraction yields of LEJ

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Yields (g/100 g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol/water (70/30)</td>
<td>15</td>
</tr>
<tr>
<td>Butanol</td>
<td>12</td>
</tr>
<tr>
<td>Ethanol-Ethyl acetate (2/1)</td>
<td>9</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (8/2)</td>
<td>1.2</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (7/3)</td>
<td>2.4</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (5/5)</td>
<td>3.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 2. Phenolic and Flavonoids content in each fraction and their antioxidant activity.

<table>
<thead>
<tr>
<th>Component (mg/g dry weight)</th>
<th>Phenolic</th>
<th>Flavonoids</th>
<th>IC50 on DPPH radical (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol-ethyl acetate (2/1)</td>
<td>28 ± 1.3</td>
<td>7 ± 0.52</td>
<td>42 ± 2.1</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (8/2)</td>
<td>2 ± 0.04</td>
<td>0.4 ± 0.03</td>
<td>83 ± 3.0</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (7/3)</td>
<td>5 ± 0.07</td>
<td>0.8 ± 0.03</td>
<td>67 ± 2.4</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (5/5)</td>
<td>8 ± 0.09</td>
<td>1.4 ± 0.08</td>
<td>55 ± 1.7</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (0/1)</td>
<td>13 ± 0.4</td>
<td>4.3 ± 0.1</td>
<td>12 ± 0.8</td>
</tr>
<tr>
<td>BHT</td>
<td>-</td>
<td>-</td>
<td>69 ± 3.2</td>
</tr>
</tbody>
</table>

DPPH radical scavenging activity

The antiradical activities of the extracts were determined using the DPPH free radical assay (figure 2) and the radical scavenging activities were expressed as the mean of the IC50 values (µg/mL). IC50 values and BHT were reported in Table 2. Our results show that the EtOH/MeOH 2:1 extract exhibits a capacity to reduce the DPPH with an IC50 of 42 µg/mL. Using this extract, the most potent fraction obtained with CH2Cl2/MeOH (0:1) shows an IC50 value about 12 µg/mL, being 3.5 times more active than the initial extract.

This result shows that there is correlation between the enrichment of phenolic and flavonoids compounds and the antiradical activity. Consequently, we can hypothesize that phenolic or flavonoids compounds might be responsible for the antiradical activity.

Table 3. Total phenolic and flavonoids content

<table>
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<tr>
<th>Solvents</th>
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<th>Total flavonoids content</th>
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<tr>
<td>Ethanol-ethyl acetate (2/1)</td>
<td>12 ± 0.4</td>
<td>109.3 ± 0.08</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (8/2)</td>
<td>13 ± 0.4</td>
<td>119 ± 0.08</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (7/3)</td>
<td>14 ± 0.4</td>
<td>129 ± 0.08</td>
</tr>
<tr>
<td>CH2Cl2-MeOH (5/5)</td>
<td>15 ± 0.4</td>
<td>139 ± 0.08</td>
</tr>
<tr>
<td>Methanol</td>
<td>16 ± 0.4</td>
<td>149 ± 0.08</td>
</tr>
</tbody>
</table>

Results

Extraction yields of plant material

Dried and powdered LEJ were extracted with MeOH/H2O 7:3 and then fractionated after that with butanol, EtOH/MeOH 2:1 and CH2Cl2/MeOH at different percentage. Table 1 summarizes the extraction yield of LEJ.

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Figure 2: Radical scavenging activities of LEJ extracts and fractions measured on DPPH.

Evaluation of the anti-inflammatory effect
To evaluate the anti-inflammatory effect, we measured the ability of these extracts and fractions to inhibit the inflammatory hG-IIA (figure 3A, 3B) and the digestive pG-IB (figure 3C) phospholipases A2.

Table 3. Inhibitory effect of LEJ extracts on hG-IIA and pG-IB phospholipases.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>IC_{50} values on hG-IIA (µg/mL)</th>
<th>IC_{50} values on pG-IB (µg/mL)</th>
<th>Inhibition specificity (IC_{50} pG-IB/IC_{50} hG-IIA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol-ethyl acetate (2/1)</td>
<td>8 ± 0.4</td>
<td>1200 ± 50</td>
<td>150</td>
</tr>
<tr>
<td>CH_{2}Cl_{2}/MeOH (8/2)</td>
<td>2300 ± 100</td>
<td>&gt; 5000</td>
<td>&gt; 2.17</td>
</tr>
<tr>
<td>CH_{2}Cl_{2}/MeOH (7/3)</td>
<td>3500 ± 120</td>
<td>&gt; 5000</td>
<td>&gt; 1.42</td>
</tr>
<tr>
<td>CH_{2}Cl_{2}/MeOH (5/5)</td>
<td>1000 ± 40</td>
<td>&gt; 5000</td>
<td>&gt; 5</td>
</tr>
<tr>
<td>CH_{2}Cl_{2}/MeOH (0/1)</td>
<td>4 ± 0.3</td>
<td>800 ± 20</td>
<td>200</td>
</tr>
</tbody>
</table>

Discussion
In this study, we targeted the extraction of phenolic and flavonoids compounds present in LEJ. In fact, several previous works described the importance of the biological functions of these molecules such as antioxidant24,30–32, anti-inflammatory33,34, anti-atherosclerotic35,36, anticancer35,37,38 and antimicrobial activities39,40. Indeed, the ethanol-ethyl acetate (2/1) extract contains 28 mg GAE/g DW of phenolic compounds and 7 mg EQ/g DW of flavonoid contents and show an impor-
tand antioxidant activity measured on DPPH with an IC50 of 42 µg/mL. These results are in agreement with those obtained by" and" who reported that there is a close relationship between phenolic and flavonoid content and the antioxidant activity in Eriobotrya japonica extracts.

On the purpose to identify natural anti-inflammatory compounds, several studies were performed using Eriobotrya japonica due to its well known potent anti-inflammatory effects and these have demonstrated that leaf of Eriobotrya japonica was able to suppress LPS-induced cytokine production in a dose dependent manner. Moreover, they have proved that water extract of Eriobotrya japonica leaves regulates production of anti-inflammatory cytokines such as TNFα, IL6 and IL8 in mast cells. We also reported in this study that the ethanol-acetate (2/1) extract of Eriobotrya japonica inhibits the pro-inflammatory PLA2 (hG-IAA) with an IC50 of 8 µg/mL. The selective inhibition was performed using the digestive PLA2 (pG-IB) and our results reveal that the EtOH/EtOAc 2:1 extract inhibits the pancreatic enzyme with an IC50 of 1200 µg/mL.

This result confirms that the extract inhibits preferentially the pro-inflammatory PLA2 with a relative selectivity factor of 150. These results have encouraged us to split over this extract. On this purpose, liquid-liquid extraction was performed using CH2Cl2/MeOH at various percentages. Obtained fractions were evaluated for their phenolic and flavonoids content in CH3CN versus pG-IB with IC50 values of 4 µg/mL and 800 µg/mL. These results have demonstrated the best capacity to inhibit hG-IIA secretory PLA2 group IIA, LEJ: leaves of Eriobotrya japonica. To that end, we performed fractionation of EtOH/EtOAc 2:1 using CH3Cl/MeOH in different proportions. The evaluation of these fractions shows that a correlation may exist between phenolic and flavonoids compounds and the anti-inflammatory and the antioxidant activities. So far we are using fraction from LEJ and its fraction; the compound responsible for the preferential inhibition of the hG-IAA PLA2 is still not identified. The efforts in purification and identification of active components from LEJ are ongoing.

Abbreviations: IC50: inhibitory concentration at 50 %, sPLA2: secreted phospholipase A2, hG-IAA: human secreted phospholipase A2 group IIA, pG-IB: pig secreted phospholipase A2 group IB, LEJ: leaves of Eriobotrya japonica, DPPH: 2,2-diphenyl-1-pircrylhydrazyl, CH3Cl: dichloromethane, MeOH: methanol, DW: dry weight, GAE: gallic acid equivalent, QE: quercetin equivalent, NaTDC: sodium taurodeoxycholate

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References