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 properties[3,5–8].

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 cells[11]. sPLA2 enzymes are a heterogenic family that are divided on 11 groups (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB)[12–14]. The sPLA2 group IIA was initially detected in synovial fluid of patients with rheumatoid arthritis[15,16]. Several studies demonstrated that the sPLA2 group IIA was involved

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in inflammatory process[17–19] and many phospholipases A2 inhibitors have been discovered and their effectiveness have been proved as a treatment of inflammatory diseases[20–22].

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 Narural 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 methanol was removed by evaporation and 250 mL of n-butanol was added. The organic phase was evaporated and the extract was dissolved in 200 mL of EtOH/EtOAc 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 phenols determination

Total phenols determination of the fractions of Eriobotrya japonica leaves extracts was determined by colorimetric assay according to the method described by [23]. 1 ml of sample at 1 mg/ml was mixed with 1 ml of Folin-Ciocalteu reagent. After 3 min of incubation, 1 ml of saturated Na_2CO_3 solution was added and the volume was adjusted to 10 ml with distilled water. The reaction mixture was kept in the dark for 90 min, after which the absorbance was read at 725 nm. The total phenolic content was determined using gallic acid as a standard.

Determination of flavonoids content

Total flavonoids were determined by following the procedure[24]. Briefly, 1 mL of aliquots of leaves extracts and fractions were placed in two test tubes, respectively. 7 mL of methanol were added to one tube. In the other one, 1 mL of 2 % ZrOCl2-8H2O and 6 mL of methanol were added. The solution was mixed again and placed into water bath at 30 °C for 1 h. The absorbance was measured at 420 nm and ΔOD was calculated. The amount of total flavonoids was calculated as a quercitin equivalent from the standard curve (figure 1), and expressed as mg quercitin/g dry leaves plant material (mg/g dry weight).

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[25–27] 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:

Scavenging effect (%) =

$$\left(1 - \frac{OD\ sample}{OD\ control}\right) X\ 100$$

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

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[28]. Briefly, the substrate consisted of 3.5 mM lecithin (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 blot. 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 Duncan test performed after analysis of variance (ANOVA).

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/EtOAc 2:1 and CH2Cl2/MeOH at different percentage. Table 1 summarizes the extraction yield of LEJ.

Table 1. Extraction yields of LEJ

Solvents	Yields (g/100 g dry weight)
Methanol/water (70/30)	15
Butanol	12
Ethanol-Ethyl acetate (2/1)	9
CH_2Cl_2-MeOH (8/2)	1.2
CH_2Cl_2-MeOH (7/3)	2.4
CH_2Cl_2-MeOH (5/5)	3.1
Methanol	2.3

Total phenolic and flavonoids content

Total phenolic content, expressed as mg GAEs/g DW and flavonoids content, expressed as mg quercitin/g DW of LEJ extracts were presented in Table 2. Results show that phenolic and flavonoids content in EtOH/EtOAc 2:1 extract were about 28 mg GAEs/g DW and 7 mg EQ/g DW, respectively. These concentrations were lower than those from E. japonica cv. Zaozhong No. 6 and E. japonica Lindl[29]. These studies show that the two species contain 47.5 and 54.9 mg GAEs/g DW as phenolic content and 109.3 and 119 mg QE/g DW as flavonoids content, respectively. Therefore, the EtOH/EtOAc 2:1 extract of LEJ was further fractionated into CH2Cl2/MeOH (8:2, 7:3, 5:5 and 0:1) soluble fractions. Results reported in Table 2 show that CH2Cl2/MeOH (0:1) extract was the richest on phenolic and flavonoids compound with 13 mg GAEs/g DW and 4.3 mg QE/g DW, respectively.

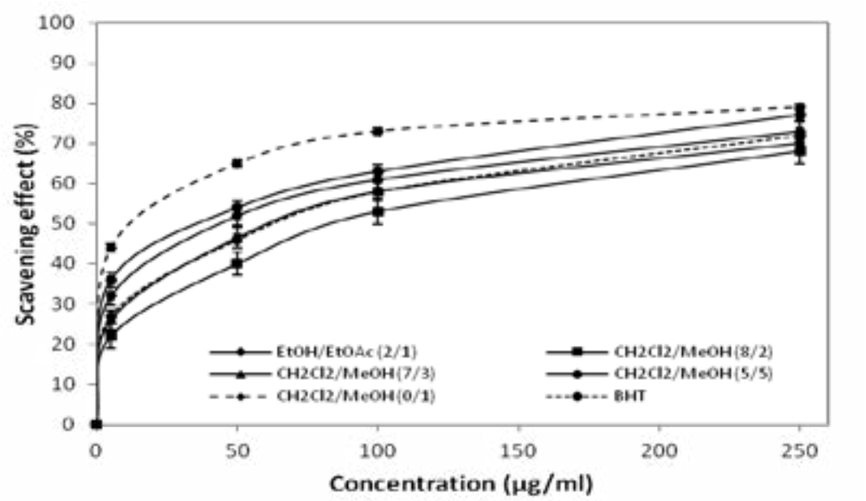
Table 2. Phenolic and Flavonoids content in each fraction and their antioxidant activity.

Fractions	Component (mg/g dry weight)		
	Phenolic	Flavonoids	IC_{50} on DPPH radical (µg/mL)
Ethanol-ethyl acetate (2/1)	28 ± 1.3	7 ± 0.52	42 ± 2.1
CH_2Cl_2-MeOH (8/2)	2 ± 0.04	0.4 ± 0.03	83 ± 3.0
CH_2Cl_2-MeOH (7/3)	5 ± 0.07	0.8 ± 0.03	67 ± 2.4
CH_2Cl_2-MeOH (5/5)	8 ± 0.09	1.4 ± 0.08	35 ± 1.7
CH_2Cl_2-MeOH (0/1)	13 ± 0.4	4.3 ± 0.1	12 ± 0.8
BHT	-	-	69 ± 3.2

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/EtOAc 2:1 extracts exhibit 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.

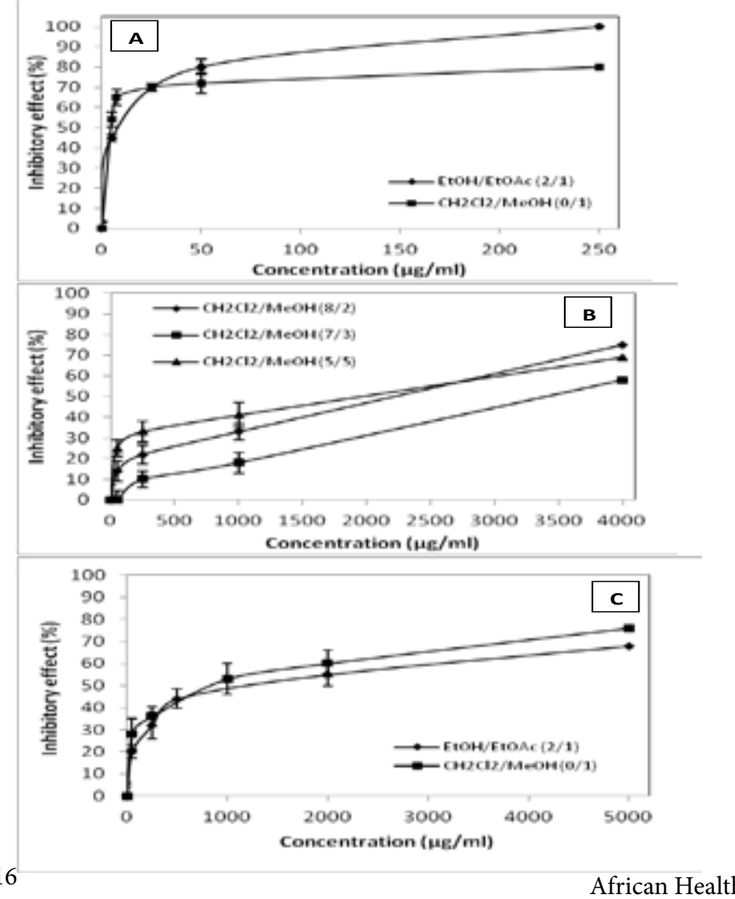
Figure 2: Radical scavenging activities of LEJ extracts and fractions measured on DPPH.



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.

Figure 3: Inhibitory effect of LEJ extracts and fractions on PLA2. A and B: pro-inflammatory PLA2 (hG-IIA), C: digestive PLA2 (pG-IB).



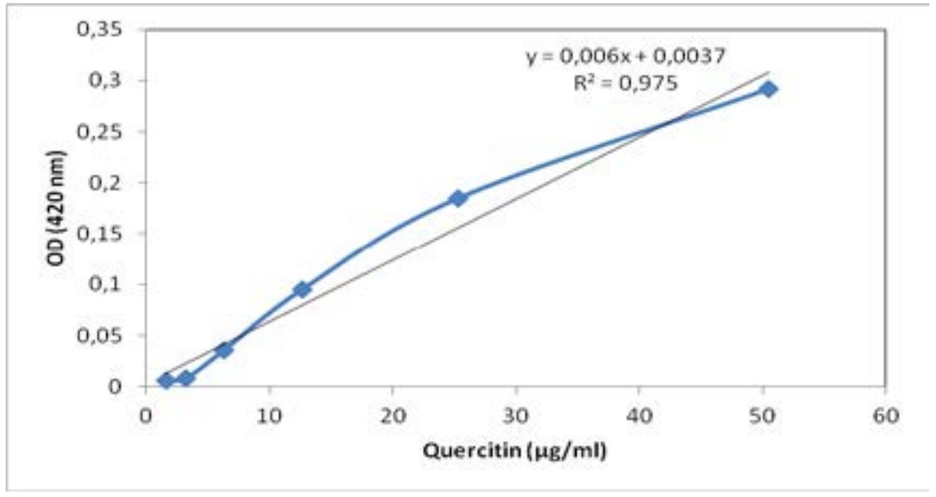
Results show that the EtOH/EtOAc 2:1 extract inhibits the hG-IIA PLA2 and the pG-IB with an IC50 of 8 µg/mL and 1200 µg/mL, respectively (Table 3). This finding proves that this extract inhibits preferentially hG-IIA with a relative specificity inhibition factor of about 150. Fractions from this extract were tested for their ability to inhibit these two PLA2 and results revealed that CH2Cl2/MeOH 0:1 fraction is the most interesting one (Table 3).

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

Fractions	IC_{50} values on hG-IIA (µg/mL)	IC_{50} values on pG-IB (µg/mL)	Inhibition specificity (IC_{50} pG-IB /IC_{50} hG-IIA)
Ethanol-ethyl acetate (2/1)	8 ± 0.4	1200 ± 50	150
CH_2Cl_2-MeOH (8/2)	2300 ± 100	> 5000	> 2.17
CH_2Cl_2-MeOH (7/3)	3500 ± 120	> 5000	> 1.42
CH_2Cl_2-MeOH (5/5)	1000 ± 40	> 5000	> 5
CH_2Cl_2-MeOH (0/1)	4 ± 0.3	800 ± 20	200

In fact, this fraction inhibits preferentially the hG-IIA enzyme with an IC_{50} of 4 µg/mL versus 800 µg/mL measured on pG-IB PLA2. To highlight the specificity inhibition of hG-IIA versus pG-IB, we calculate the specificity factor $\frac{IC50\ (pG-IB)}{IC50\ (hG-IIA)}$ which is around 200. This value indicates that the inhibitory potency of this fraction toward hG-IIA is 200 times higher than its toward pG-IB. This fraction was likely able to inhibit preferentially the inflammatory PLA2 (hG-IIA) and not the digestive one (pG-IB). Moreover, we can strongly suggest that phenolic or flavonoid compounds in CH2Cl2/MeOH 0:1 were responsible for the hG-IIA inhibition.

Figure 1: Standard curve of quercitin



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 antioxidant[24,30–32], anti-inflammatory[33,34], anti-atherosclerotic[35,36], anticancer[35,37,38] and antimicrobial activities[39,40]. 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-

tant antioxidant activity measured on DPPH with an IC50 of 42 µg/mL. These results are in agreement with those obtained by[41] and[24] 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[42] and these have demonstrated that leaf of Eriobotrya japonica was able to suppress LPS-induced cytokine production in a dose dependent manner. Moreover,[8] they have proved that water extract of Eriobotrya japonica leaves regulates production of pro-inflammatory cytokines such as TNFα, IL6 and IL8 in mast cells. We also reported in this study that the ethanol-ethyl acetate (2/1) extract of Eriobotrya japonica inhibits the pro-inflammatory PLA2 (hG-IIA) with an IC_{50} 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 IC_{50} 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 CH_2Cl_2/MeOH at various percentages. Obtained fractions were evaluated for their phenolic and flavonoids content and their ability to possess antioxidant and anti-inflammatory activities. Results presented in Table 2 show that CH_2Cl_2/MeOH 0:1 fraction was the richest on phenolic and flavonoids content with values of 13mg EAG/g DW and 4.3 mg EQ/g DW, respectively, and with the most antioxidant effect (IC_{50} = 12 µg/mL). In the same way, this fraction has demonstrated the best capacity to inhibit hG-IIA versus pG-IB with IC50 values of 4 µg/mL and 800 µg/mL, respectively. These results suggest that the phenolic and flavonoids compounds in CH_2Cl_2/MeOH 0:1 are responsible for preferential inhibition of hG-IIA compared to the digestive pG-IB one.

Several studies investigated medicinal plants for their potent natural therapeutic virtues and only few of them were described for their capacity to inhibit the inflammatory PLA2 enzyme. The ethanol extract of the stem of Sinomenium acutum, Spatholobus suberectus and Trachelosermum jasminoide show IC_{50} values of 112, 54 and 33 µg/mL, respectively[43]. Compared to these works, the fractions that we obtained are more efficient to inhibit the pro-inflammatory PLA2 with an IC50 of 4 µg/ml.

Conclusion

The aim of the present study was to evaluate the anti-inflammatory and the antioxidant activities of phenolic and flavonoids content in Eriobotrya japonica leaves. To that end, we performed fractionation of EtOH/EtOAc 2:1 using CH_2Cl_2/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 extract from LEJ and its fraction; the compound responsible for the preferential inhibition of the hG-IIA PLA2 is still not identified. The efforts in purification and identification of active components from LEJ are ongoing.

Abbreviations: IC_{50}: inhibitory concentration at 50 %, sPLA2: secreted phospholipase A2, hG-IIA: human secreted phospholipase A2 group IIA, pG-IB: pig secreted phospholipase A2 group IB, LEJ: leaves of Eriobotrya japonica, DPPH: 2,2-diphényl 1-picrylhydrazyl, CH_2Cl_2: dichloromethane, MeOH: methanol, DW: dry weight, GAE: gallic acid equivalent, QE: quercitin equivalent, NaTDC: sodium taurodeoxycholate

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