Inhibition of Candida rugosa Lipase by Secondary Metabolites Extracts of Three Algerian Plants and their Antioxydant Activities

K. Benarous, A. Djeridane, A. Kamel and M. Yousfi

Abstract: Lipase inhibitors have generated a great interest because they could help in the prevention or the therapy of lipase-related diseases. Therefore, the aim of this work was to evaluate the inhibitory effect of secondary metabolites extracts such as phenolic compounds and saponins of three Algerian medicinal plants: Achillea santolina, Inonotus hispidus and Zizyphus lotus, indeed their antiradicalaire activity using DPPH• (1, 1-diphenyl-2-picryl-hydrazyl). The phenolic extracts have shown a strong antiradicalaire activity than the saponin extracts with EC50 values ranged from 6 to 11 µg/ml and from 51 to 82 µg/ml, respectively. The enzymatic inhibition produced by these plant extracts is described here for the first time. The results have shown that the phenolic extracts are more potent than the saponin extracts with Ki values ranged from 0.011 mg/ml to 0.027 mg/ml for phenolic extracts, and ranged from 0.071 mg/ml to 0.69 mg/ml for saponin extracts. The nature, mechanism and possible physiological relevance of lipase inhibition by extract components are discussed.

Keywords: Achillea santolina, Inonotus hispidus, Zizyphus lotus, Candida rugosa lipase, inhibition, saponins extracts, phenolic extracts, DPPH•.

1. INTRODUCTION

Lipase is an enzyme that hydrolyzes triacyl glycerols (TGs). The digestion and absorption of natural lipids begin with hydrolysis by pancreatic lipase. The activity of this enzyme greatly affects the metabolism of fat and the concentration of TAG in blood [1]. Obesity is caused as a result of an imbalance between energy intake and expenditure. Excess energy is stored in fat cells, which enlarge or increase in number.

Human obesity is one of the most serious health problems. Obesity is associated with an increased risk of several serious diseases including hypertension, coronary heart disease, type II diabetes, stroke, osteoarthritis and cancer. Consumption of the dietary fat is an important contributor to human obesity. Gastric and pancreatic lipases (PL) are the principal lipolytic enzymes in the gastrointestinal (GI) tract that are responsible for the hydrolysis of triacyl glycerides (TAG). Thus, inhibition of lipase in the GI tract reduces the amount of fat that can be absorbed [2]. It is generally thought that a potent and specific inhibitor of PL could be useful in the treatment of obesity [3]. Orlistat, a potent inhibitor of PL and one of the best-selling drugs worldwide, has proved useful in the treatment of obesity, but side effects due to its potency, such as fecal incontinence, have also arisen [3]. Indeed, Propionibacterium acnes lipase and its inhibition by antiaacne compounds have been studied because the fatty acids produced by P. Acnes lipase activity on sebaceous triglycerides induce severe inflammation. Tetracyclines have been the most common systemic therapy for acne due to their beneficial clinical effects, their inhibition of lipase activity of P. acnes as well as their inhibition of P. acnes chemotaxis [4]. Therefore, research on new lipase inhibitors for the therapy of these diseases and also for other pathologies like ulcer and cutaneous diseases [5] has generated a great interest. Recently, inhibitors of lipase and lipid absorption have been isolated from natural sources with the aim of preventing and treating metabolic syndrome. For our knowledge, No report has been found for evaluating the inhibitory activity of these three Algerian medicinal plants. In the present study, we report the inhibitory effect of phenolic and saponins extracts of three Algerian medicinal plants on Candida rugosa lipase for the first time.

2. MATERIALS AND METHODS

2.1. Materials

Achillea santolina, Inonotus hispidus and Zizyphus lotus were collected in April 2009 in Laghouat City, South of Algeria. The plant material (Achillea santolina and Zizyphus lotus) was identified by two of the authors (Benarous and Yousfi). Inonotus hispidus was identified by Bernard Duhem, Museum National d'Histoire Naturelle, Laboratoire de Cryptogamie, 12 Buffon street, 75231 Paris, FRANCE. Voucher specimens were deposited in the laboratory of fundamental sciences of Laghouat University. Candida rugosa lipase, p-nitrophenyllaurate, 2,2-diphenyl-1-picryl-hydrazyl (DPPH•) and all other reagents were purchased from Sigma-Aldrich. All other chemicals and solvents used were of analytical grade.

2.2. Phytochemical Tests

Before the extraction procedure, phytochemical tests have been realized using standard phytochemical procedures [6-11] to prove that the plants mentioned above contain...
phenolic compounds and saponins. These tests have confirmed that the three Algerian plants Achillea santolina, Inonotus hispidus and Zizyphus lotus contain phenolic compounds which are mentioned in previous works [12, 13]. In this work, Saponins have been found in the fungus Inonotus hispidus and Achillea santolina for the first time. Indeed, we have found that Algerian Zizyphus lotus is rich on saponins which is mentioned for the Tunisian plant by Renault et al. (1997) [14].

2.3. Preparation of Plant Extracts

The plants were air dried and ground into fine powder. The powdered material (5 g) was extracted for 30 h with 100 ml of alcohol mixture: 5/4/1 (v/v/v) ethanol/ methanol/water at room temperature. After removal of methanol/ethanol under reduced pressure in a rotary evaporator at 40°C, the remaining aqueous solution of the extraction is defatted twice with petroleum ether to remove lipids and pigments. Then, the aqueous solution obtained is extracted with ethyl acetate. The ethyl acetate (Et-O-Ac) fraction is dried by addition of a sufficient amount of anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The residue is dried, dissolved in 10 ml of methanol and kept at +4°C, this fraction contain phenolic compounds. The aqueous solution obtained is extracted twice with butanol. The butanol (BuOH) fraction is evaporated to dryness using a rotary evaporator. The residue is dried, dissolved in 10 ml of aqueous ethanol 50 % and kept at +4°C, this fraction contain saponins. All of these extracts have been tested phytochemically using the same procedure mentioned in section 3.1 to prove the existence of phenolic compounds in Et-O-Ac fractions and saponins in BuOH fractions.

2.4. Quantification of Total Phenols, Flavonoids and Saponins

The amount of total phenolics in plant extracts was estimated by the Folin–Ciocalteu reagent [15, 16], which is considered as the best method for total phenolics (included tannins) determination [17]. The total phenolic content of the ethyl acetate and butanol extracts was expressed as gallic acid equivalents (GAE). Whereas, the total flavonoid content in the ethyl acetate and butanol extracts was determined by a slightly modified version of the method of Zhishen et al. (1999) and Dewanto et al. (2002) [18, 19]. Flavonoid contents were calculated using a standard calibration curve, prepared from quercetin. The total saponins content of the butanol extracts was estimated by the method of Hiai et al. (1976) [20] and was expressed as digitonin equivalents.

2.5. Free Radical Scavenging DPPH•

The method is based on the reduction of alcoholic DPPH• solutions in the presence of a hydrogen donating antioxidant. DPPH• solutions show a strong absorption band at 515 nm with a deep violet color. The absorption vanishes and the resulting decoloration is stochiometric with respect to degree of reduction. The remaining DPPH•, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant [21-23]. Radical scavenging activity of plant extracts against stable DPPH• (2,2-diphenyl-2- picrylhydrazyl) was determined spectrophotometrically by the slightly modified method of Brand Williams et al. (1995) [24]. 1 ml of various concentrations of sample solution diluted in methanol were added to 1 mL of methanolic DPPH• radical solution (250 µM). The mixture was then shaken vigorously and allowed to stand at room temperature in obscurity for 30 min. The decrease in absorption was measured at 515 nm against a blank. Inhibition of DPPH• free radical in percent (I %) was calculated as follows:

\[ I\% = \left( 1 - \frac{A_{sample}}{A_{blank}} \right) \times 100 \]

Where \( A_{blank} \) is the absorbance of the control reaction (containing all reagents except the test compound) and \( A_{sample} \) is the absorbance of the test compound. The antioxidant activity of the extract was expressed as an EC50 value defined as the concentration (in µg/ml) of the extract that inhibited the formation of DPPH• radicals by 50%. Triplicate measurements were carried out and their activity was calculated by the percentage of DPPH• scavenged. The DPPH• radical scavenging activity obtained for each plant extract was compared with that of Vitamin C, Trolox, gallic acid and quercetin.

2.6. Assay of Candida rugosa Lipase Inhibition

The method of Prim et al. (2000); Prim et al. (2003) and Ruiz et al. (2006) [5, 25, 26] was adopted for assays inhibition with a lot of modifications. The non-colored substrate p-NPL was dissolved at 10 mM in 2-propanol by vortex for 3 min. A 1:10 (v/v) dilution in phosphate saline buffer with pH 7 was prepared with gentle agitation until an optically clear and stable emulsion was achieved. Briefly, 100 µl of enzyme solution (2.5 mg/ml) were added into 100 µl of diluted plant extracts (as inhibitor) which it was previously preincubated for 15 min at 37 °C. Then, 900 µl of the substrate mixture were added, this reaction mixture were incubated for 15 additional minutes at 37 °C and the reaction was terminated by addition of 100 µl of 0.1 M Na2CO3. Released p-nitrophenol (the yellow-colored product of the reaction) was immediately determined by measuring the absorbance at λ = 405 nm in a Spectrophotometer (Jenway 6405). All assays were performed within the linear range of absorbance of the p-nitrophenol (p-NP) calibration curve, which was obtained under the same assay conditions. One unit of activity was defined as the amount of enzyme that released 1 µmol of p- NP per minute under the assay conditions described.

Inhibition kinetics were performed using varying concentration of phenolic and saponin extracts in the assay mixture. Reciprocal initial velocity plots versus inhibitor concentration were used to determine the mode of inhibition. The inhibition constants (Ki) were calculated according to the Dixon model from curve fits using Microsoft Excel 2010. The concentrations yielding a lipase inhibition of 50% (IC50) were calculated from the inhibition vs. plant extract concentration curves by regression analysis. All experiments were done at least in triplicate.
3. RESULTS AND DISCUSSION

3.1. Quantification of Total Phenols, Flavonoids and Saponins

Solvent extraction is a commonly used method to obtain secondary metabolites from plant materials. However, it has been suggested that no single solvent can extract all the secondary metabolites from plants because of its variation in solubility and polarity [27-30]. For this reason and after many experiments we have used the solvent mixture ethanol/methanol/water with 5/4/1 (v/v/v). The extraction yields in both fractions (Et-O-Ac and BuOH) ranged from 1.46 to 9.21 % where BuOH extracts are showing a higher ratio than the Et-O-Ac extracts (Table 1). This result could interpret by the polarity, high molecular weight and solubility of phenolic compounds, flavonoids and saponins to be extracted with BuOH solvent [31-34].

The amount of total phenolic varied in the three plants with the both solvent extraction and ranged from 2.66±0.18 to 6.35±0.3 mg GAE/g of dry material for Et-O-Ac fractions, and ranged from 0.26±0.04 to 0.82±0.03 mg GAE/g of dry material for BuOH fractions (Table 1). The Et-O-Ac extracts have shown higher total phenolic and total flavonoids content than the BuOH extracts. We have marked that even the BuOH fraction showed the higher ratios of dry residue, it showed the lower quantities of total phenolic and flavonoid content than the Et-O-Ac fractions which prove that the BuOH has extracted the compounds with high molecular weight but without phenolic structures, we admit that these molecules are saponins. It has been noted that the amount of total phenolic compounds in Asteraceae varieties which is represented here by Achillea santolina is higher than the other families Rhamnaceae and Basidiomycetes. However, this phenolic content of Achillea santolina is lower than other plants in the same family such as: Artemisia campestris with 20.38±0.30 mg GAE/g dw and Globularia alypum with 21.54±0.81 mgGAE/g dw. But this amount is higher than Artemisia arboresens with 3.42±0.50 mg GAE/g dw and Ruta Montana with 3.13±0.30 mg GAE/g dw [35, 36]. The total phenolic content in the plants tested in this study was lower than those reported for the most of other Asian medicinal and common dietary plants [37-41].

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolic. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [36]. In this study, the content of flavonoids (mg/g), in quercetin equivalent which varied from 0.1 to 0.97 has shown higher values in the Et-O-Ac extracts than the BuOH extracts. Therefore, these amounts presented lower values than the most of African and Asian medicinal plants [35-37].

As we have already mentioned that these plants contain saponins, we have estimated the quantity of saponins in the BuOH fractions. Saponins are widely distributed among plants and have a wide range of biological properties. The more recent investigations and findings on their biological activities were summarized [42]. The amount of saponins of the three plants was ranged from 3.84±0.38 to 123.7±5.69 mg DE/g dw. Total saponins content has presented the highest value for the Zizyphus lotus with 123.7±5.69 mg DE/g dw which is higher than Tribulus terrestris [43], and the lowest value for Inonotus hispidus with 3.84±0.38 mg DE/g dw but it is higher than Ipomoea batatas [44].

These saponins quantities in the three Algerian plants are higher than the total phenolic and flavonoids quantities in both fractions (Et-O-Ac and BuOH).

3.2. Free Radical Scavenging DPPH•

The aim of finding potent antioxidants is to reveal a relationship between antiradicalaire activity and lipase inhibitory activity. The 1,1-diphenyl-2-picrylhydrazine (DPPH•) radical scavenging is one of the most extensively used antioxidant assays for plant samples [45]. The phenolic and saponins extracts were evaluated for their antiradicalaire activity using a chemical method (DPPH• assay). The DPPH• was used in this assay to assess the free radical scavenging (antioxidant) properties of plant extracts and Trolox, vitamin C, gallic acid and quercetin, well known as a potent antioxidant controls [46-48]. A parameter introduced recently for the interpretation of the results from the DPPH• method is the “efficient concentration” or EC50 value. This is defined as the concentration of the substrate that causes 50% loss of the DPPH• activity. The EC50 values were calculated by the linear regression method of plots of the percent of antiradical activity against the concentration of the tested compounds [36]. The concentration of inhibitors has been calculated in µg/mL. The results obtained are summarized in (Table 2). All the Et-O-Ac extracts showed a beneficial effect against free radical damage compared with the antioxidant controls but total saponin extracts in BuOH

Table 1. Extraction Yield, Total Phenols, Total Flavonoids and total Saponins Contents for the Studied Plants mg/g Dry Weight

<table>
<thead>
<tr>
<th>Plant</th>
<th>Part</th>
<th>Fraction</th>
<th>Extraction Yield %</th>
<th>Total Phenols (mg GAE /g dw)</th>
<th>Total Flavonoids (mg QE /g dw)</th>
<th>Total Saponins (mg DE /g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achillea santolina</td>
<td>Leaves and flowers</td>
<td>Et-O-Ac</td>
<td>5.20</td>
<td>6.35±0.30</td>
<td>0.97±0.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BuOH</td>
<td>8.90</td>
<td>0.82±0.03</td>
<td>0.28±0.02</td>
<td>21.90±2.09</td>
</tr>
<tr>
<td>Zizyphus lotus</td>
<td>Leaves</td>
<td>Et-O-Ac</td>
<td>4.94</td>
<td>6.09±0.31</td>
<td>0.83±0.01</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BuOH</td>
<td>9.21</td>
<td>0.63±0.38</td>
<td>0.69±0.04</td>
<td>123.70±5.69</td>
</tr>
<tr>
<td>Inonotus hispidus</td>
<td>fungus</td>
<td>Et-O-Ac</td>
<td>1.46</td>
<td>2.66±0.18</td>
<td>0.53±0.01</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BuOH</td>
<td>4.20</td>
<td>0.26±0.04</td>
<td>0.10±0.00</td>
<td>3.84±0.38</td>
</tr>
</tbody>
</table>

fraction exhibited a moderate antiradical activity than the phenolic compounds (gallic acid and quercetin) and the synthetic antioxidants (trolox and vitamin C). It has been reported in many researches that saponins showed a moderate antioxidant activity [44, 49, 50]. The phenolic compounds and the flavonoids have registered a higher activity than the most medicinal plants mentioned in Djeridane et al. [2010] [36] such as: Artemisia campestris with EC50 of 09.61 ± 0.02 µg/ml, Cleome arabica 13.15 ± 0.01 µg/ml, Deverra scoparia 14.70 ± 0.03 µg/ml and Ononis angustissima 20.83 ± 0.08 µg/ml and Teucrium polium flavonoids in Sharififar et al. [2009] [51]. Furthermore, the phenolic compounds of Inonotus hispidus have shown a powerful activity and we suggest that it is due to the potent phenols such as Hispolon and Hispidin as registered by Yousfi et al. [2009] [13]. It can be observed that the phenolic content of the extracts had no significant correlation with their antiradical activity as measured by the DPPH• assay (R²<0.1).

### 3.3. Assay of Candida rugosa Lipase Activity

The effect of several polyphenol, flavonoids and saponins extracts on C. rugosa lipase was analyzed to evaluate their potential as antilipase drugs. Recently, inhibitory effects of these natural secondary metabolites on pancreatic lipase were reported to be involved in anti-obese effects such as flavonoids (e.g. Mangiferin, Hesperidin) [52-54] and saponins (e.g. the asaponins, chikusetsusaponins) [55, 56]. In this study, Candida rugosa lipase has shown normal Michaelis kinetics on p-nitrophenyllaurate (p-NPL) and for each extract the IC50 values were calculated from the plot of the enzyme activity as a function of phenolics and saponin concentration. Among the extracts tested, Et-O-Ac extracts were more active than BuOH extracts and the only inactive one was the BuOH extract of Inonotus hispidus (Table 3, Fig. 1); the different inhibition produced by these extracts is probably related to their substances with different chemical structures and physicochemical properties.

### Table 3. IC50, Ki Values and Type Inhibition for Each Extract

<table>
<thead>
<tr>
<th>Plant</th>
<th>Organic Fraction</th>
<th>Ext</th>
<th>IC50 (mg/ml)</th>
<th>Ki (mg/ml)</th>
<th>Inhibition Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Achillea santolina</strong></td>
<td>Ac-O-Et</td>
<td>dry res</td>
<td>2.32±0.25</td>
<td>0.14±0.01</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phe</td>
<td>0.37±0.00</td>
<td>0.02±0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>dry res</td>
<td>4.83±0.15</td>
<td>0.30±0.00</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phe</td>
<td>0.05±0.00</td>
<td>0.00±0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>sap</td>
<td>1.19±0.03</td>
<td>0.07±0.00</td>
<td></td>
</tr>
<tr>
<td><strong>Zizyphus lotus</strong></td>
<td>Ac-O-Et</td>
<td>dry res</td>
<td>3.70±0.00</td>
<td>0.22±0.00</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phe</td>
<td>0.45±0.00</td>
<td>0.03±0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>dry res</td>
<td>8.27±0.23</td>
<td>0.50±0.01</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phe</td>
<td>0.06±0.00</td>
<td>0.01±0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>sap</td>
<td>11.53±0.46</td>
<td>0.69±0.03</td>
<td></td>
</tr>
<tr>
<td><strong>Inonotus hispidus</strong></td>
<td>Ac-O-Et</td>
<td>dry res</td>
<td>2.22±0.03</td>
<td>0.04±0.00</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phe</td>
<td>0.56±0.02</td>
<td>0.01±0.00</td>
<td></td>
</tr>
</tbody>
</table>

Dry res: dry residue, phe: phenols, sap: saponins
Inhibition of Candida rugosa Lipase by Secondary Metabolites

Many scientific researches and studies have been done for evaluating anti-obesity and anti-acne effects of the medicinal plants extracts on lipase activity aiming to find a new natural drug for both diseases without secondary effects. In this study, we have done the same and we want to compare our plant extracts with those works (Table 4). In the aim to show which plant family contains the most potent species as lipase inhibitors, we have grouped in Table 4 from different literature sources the most powerful plants with their family and their IC50, we reported that in the family of Compositae the Et-O-Ac extract of Achillea santolina have presented a potent lipase inhibition than the 50 % EtOH extract of Gynura pseudochina which it is already used for the acne treatment (Table 4). For Inonotus hispidus and Zizyphus lotus, we have not found any researches for species in the same families but compared with the other plants mentioned in Table 4, our plants extracts are less potent lipase inhibitors than the other species of families: Fabaceae, Zingiberaceae, Sapindaceae, Moraceae, Saxifragaceae, Lamiaceae and Araliaceae. On the other hand, the polyphenols of the Et-O-Ac extracts of A. santolina, Z. lotus and I. hispidus are very powerful lipase inhibitors than polyphenols of Salacia reticulate which it is used for obesity treatment, catechin and kaempferol. Furthermore, the saponins of the BuOH extracts of A. santolina and Z. lotus are more effective inhibitors than cyclocariosides (Dammarene saponins) of Cyclocarya paliurus but less effective than gypsogenin 3-O-b-D-glucuronide (triterpenoid saponin) of Acanthopanax senticosus. Among all extracts, there is no extract more active than the anti-obese drug Orlistat.

We have tried to propose the active molecules in the previous extracts basing on the literature; the Et-O-Ac extract of A. santolina is rich with flavonoids such as quercetin and luteolin [57]. Quercetin is known as a potent lipase inhibitor (Table 4) so we suggest that this molecule is maybe responsible for the inhibition. The BuOH extract of Z. lotus is probably containing the dammarane saponins [58] which are known for their inhibitory effect and we suggest that they may be responsible for this inhibition. The Dixon plots of inhibition of Candida rugosa lipase for each extract have shown one inhibition type which it is uncompetitive. The uncompetitive inhibition occurs when the inhibitor binds to a site distinct which only becomes available after the substrate (S1) has bound to the active site of the enzyme. This inhibition is most commonly encountered in multi-substrate reactions [59]. In fact, we suggest that C. rugosa lipase contains another site than the active one which it is constituted of characteristics amino acids; this site may fixe the inhibitor changing the protein conformation and inhibiting the reaction catalysis.

CONCLUSION

All the plants presented in this work are already used in Arab traditional medicine for treatment of diseases but it is the first time that they have been studied for their anti-obesity, anti-acne and antioxidant effects. The results demonstrated that extracts showed certain activities against lipase, and were efficient free radical scavengers. No relation has been found between the antioxidant activity and the antilipase effect. The results from this study give a scientific support to the use of these plants in folklore medicine for the treatment of obesity and acne, and show for the first time, the potential role of lipase inhibition in its activity. For this, further studies should be achieved to identify the active molecules responsible for the C. rugosa lipase inhibition and to explain their reaction mechanism. Indeed, identifying the
active molecules for the antioxidant activity aiming to introduce these natural compounds in the drugs for the treatment of diseases mentioned above.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES


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