SUPPLEMENTARY MATERIAL

Essential oil composition and biological activities of *Ononis alba* Poir (Fabaceae)

The phytochemical and biological properties of *Ononis alba* Poir L. (Fabaceae) were investigated for the first time in this study. The chemical composition of the essential oil obtained from the aerial parts was analysed by GC-MS. The phenolic contents of extracts obtained with different solvents were determined by the Folin-Ciocalteu assay and the antioxidant activity was evaluated through DPPH and CUPRAC methods. The inhibitory potential of these extracts was carried on α-amylase and α-glucosidase, whereas the antimicrobial effect was performed against some bacteria and fungi through the well diffusion method. Ketones and carboxylic acids were the main essential oil constituents. The highest total phenolic and flavonoid content as well as the best antioxidant capacity were noticed on the *n*-butanol extract. All the extracts showed a greater efficiency than acarbose in the inhibition of α-amylase. On the other hand, they demonstrated a mild inhibition effect against *Staphylococcus aureus* and *Fusarium oxysporum*.

Keywords: Antimicrobial activity; Antioxidant activity; Enzyme inhibitory effect; Essential oil; Extracts; *Ononis alba* Poir.

Results and discussion

1. *In vitro enzyme inhibition assays*

Phenolic components are known to inhibit certain carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase, due to their ability to bind to proteins (Hanamura et al. 2005; Bothon et al. 2013). As alternative antidiabetics, several α-amylase and α-glucosidase inhibitors have recently been isolated from medicinal plants (Xiao et al. 2013). The inhibitory activities of the α-amylase and α-glucosidase of the *O. alba* dichloromethane extract, ethyl acetate and *n*-butanol extract are presented in Table S3. No absorbance was measured in case of α-glucosidase which can be interpreted as an absence of effects against this enzyme. On the other hand, the obtained values demonstrated a significant reduction in α-amylase activity with all extracts. Comparatively to the reference compound acarbose, whose IC_{50} value was estimated at 365.09 ± 10.7 μg/ml, the *O. alba* extracts showed a significantly higher efficacy (around 15 to 60 fold higher). The most important inhibition was given by the *n*-butanol extract with an IC_{50} value less than 6.25 μg/ml, followed by ethyl acetate extract (IC_{50} : 17.80 ± 4.32 μg/ml) and finally dichloromethane extract (IC_{50} : 20.24 ± 0.3 μg/ml).

Our results showed a significant lower IC_{50} values than those reported by (Laoufi et al. 2017) who used *O. angustissima* extracts on these enzymes (IC_{50} values of the extracts between 2010 and 2880 μg/ml and acarbose of 44 μg/ml). These results suggest that the inhibitory effect obtained against α-amylase activity may be exerted by one or more active compounds occurring in all the studied extracts at different concentration. According to previous studies (Kim et al. 2000; Tadera et al. 2006; Williams 2013), these bioactive agents can be flavonoids which constitute a major group of polyphenolic compounds where the inhibitory activity depend on their structure, number and the position of their hydroxyl groups in the molecule (Tadera et al. 2006).

2. *Antimicrobial activity*

The well diffusion technique was used to evaluate the antimicrobial activities of the extracts. Table S2 (Supplementary material) summarizes the microbial sensitivity of three microorganisms: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *F. oxysporum* tested using a concentration of 16 mg/ml of different *Ononis* extracts. According to the obtained data, the dichloromethane and *n*-butanol extract mildly inhibited the growth of *S. aureus*, with an inhibition zone diameter of 10–13 mm while no inhibition was observed using the ethyl

acetate extract on all tested strains. In the case of *E. coli*, no growth inhibition zone was observed around the wells of all extracts, which explain the resistance of this strain. On the other hand, the inhibitory activity of the different extracts against the fungal strain *F. oxysporum* was determined by measuring the radial growth of the fungus on a PDA medium. From the results, it can be noted that there was a decrease in the radial growth of the *F. oxysporum* using all extracts. At a concentration of 16 mg/ml, the dichloromethane, *n*-butanol and ethyl acetate extracts have not shown an important antifungal activity exhibiting low inhibition percentages of 9.84; 6.81 and 3.78% respectively.

Experimental section

1. Plant material

The aerial parts of *O. alba* were collected in June 2018 at Chemini region of Bejaia Province, situated in the north-east of Algeria. A voucher specimen (Number N° KR0006) was identified by Dr. K. Rebbas from Natural and Life Sciences Department, University of M'sila, Algeria, and deposited at the Herbarium of M'sila University.

2. Essential oil extraction

Aerial parts of *O. alba* (100 g) were cut into small pieces and submitted to hydrodistillation in a Clevenger apparatus using 3 L of deionized water for 3 h. The yellow colored essential oil were dried over anhydrous sodium sulfate Na_2SO_4 and stored in sealed vials protected from the light at 20 °C before chemical investigations.

3. Extracts preparation

Air-dried powdered aerial parts (Leaves and flowers) of *O. alba* (70 g) were extracted 3 times with a solution of 70% methanol during 24 h. The combined extracts were evaporated under reduced pressure and temperature. The residue was dissolved in 150 ml of hot distilled water, and left in cold overnight. After filtration, the aqueous extract was successively extracted by three solvents with increasing polarity (dichloromethane, ethyl acetate, *n*-butanol) and evaporated under reduced pressure, then dried and stored before assays.

4. GC-MS analysis of essential oil

Chemical composition of the essential oil was determined using Agilent 6890N gas chromatograph, coupled with a 5973N mass spectrometer, operating in the EI mode at 70 eV, equipped with an apolar capillary column HP-5MS (5% phenyl methyl silicone and 95% dimethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 µm film thickness) (J & W Scientific, Folsom). The carrier gas used was helium at a flow rate of 1.0 ml/min. The temperature gradient applied for the analysis was as followed: initial temperature 60°C for 5 min, then increase up to 220°C with a ramp at 4°C/min, from 220°C to 280°C at 11°C/min, held for 15 min and finally up to 300°C at 11°C/min, held for 5 min. Two µL were injected; with a split injection conducted with a ratio split of 1:50. The injector as well as the transfer line were set at 280°C. The scan time was 75 min and the mass range was from *m*/*z* 29 to 400. The identification of the essential oil constituents was based on their retention indices (RIs) and the comparison of their mass spectra (MS) with those reported in libraries such as Adams (2007), FFNSC2 (2012), WILEY 275 and NIST17 (2017). Relative peak area percentages were extracted for each peak from the total areas in the chromatograms without the use of correction factors.

5. TPC and TFC determination

The total Phenol Content were determined using the Folin-Ciocalteu assay, following the procedure described by (Singleton et al. 1999), with minor modifications. Gallic acid was used as standard for calibration while the total flavonoids content was also determined using an assay according to (Topçu et al. 2007) and quercetin as standard for calibration .

6. Antioxidant activity assessment

6.1. Free radical scavenging activity (DPPH assay)

The DPPH assay was determined by the method described previously with some modifications (Blois 1958; Tel et al. 2012). An aliquot of 40 µl of the sample (extracts and standards) at various concentrations was added to 160 µl of the methanolic solution of DPPH (0.1 mM) in a 96-well microliter plate. Blanks were prepared using the solvent in addition to the DPPH reagent. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm using a microplate reader. Butylhydroxyltoluene (BHT), butyl hydroxyanisole (BHA) and α-tocopherol were used as standards for activity comparison. The

free radical scavenging activity of each solution was calculated as percent inhibition according to the following equation:

$$\%Inhibition = [(A_{blank}-A_{sample})/A_{blank}] \times 100$$

Where, A_{blank} and A_{sample} are the absorbance of initial and remaining concentrations of the DPPH in the presence of samples and standards, respectively. The extract concentration providing 50% radical scavenging activity (IC_{50}) was calculated from the graph of DPPH radical scavenging effect percentage and extract concentration.

6.2. Cupric reducing antioxidant capacity (CUPRAC assay)

The CUPRAC was determined according to the method described previously with minor changes (Apak et al. 2004; Tel et al. 2012). Fifty µl of neocuproine solution (7.5 mM) and 60 µl of acetate buffer solution (1 M, pH 7.0) were added to 50 µl of Cu (II) solution (10 mM). Forty µl of the sample solutions at different concentrations were added to the above mixture. After 60 min, the absorbance at 450 nm was recorded against a reagent blank using a 96-well microplate reader. The results were given as $A_{0.5}$ (µg/ml) indicating half absorbance intensity (corresponding to the concentration) and were compared with those of the standards BHA and BHT.

7. Enzyme inhibitory activities

7.1. Inhibitory effect on α-amylase activity

The α-amylase inhibitory activity of extracts was determined using the Iodine / Potassium Iodide Method (IKI) (Zengin et al. 2014). A volume of 25 µl of the methanolic solution of extracts was mixed with 50 µl of a solution of α-amylase (1U) (previously prepared in phosphate buffer 6 mM, pH 6.9) and placed in the microplate wells; then incubated for 10 min at 37°C. After incubation, the reaction was stopped by adding 50 µl of 0.1% starch solution. A blank was prepared and incubated under the same conditions. The reaction was then stopped by adding 25 µl of 1 M HCl, and then 100 µl of the potassium iodine-iodide solution were added to the mixture. Absorbance was measured by spectrophotometer at 630 nm. The absorbance of blank was subtracted from that tested with the sample and the inhibitory activity of α-amylase was expressed as percentage, compared to acarbose (Randhir and Shetty, 2007; Zengin et al., 2014) calculated according to the formula:

$$\% \text{ Inhibition} = [(A_C - A_S) / A_C] \times 100$$

Where A_C is the absorbance of the control (100 % enzyme activity) and A_S is the absorbance of the tested sample (plant extract or acarbose).

7.2. Inhibitory effect on α-glucosidase activity

The inhibitory activity of extracts against α-glucosidase was determined by the method described by (Sinéad et al. 2014). For each extract, 50 µl of the methanolic extract solution was mixed with 100 µl of α-glucosidase solution (0.1U / ml) prepared in advance in phosphate buffer (pH 6.9; 100 mM). To the mixture, 50 µl of 5 mM substrate solution prepared in phosphate buffer (pH 6.9, 100 mM) were added, and then incubated for 10 min at 37°C. After incubation, the reaction was stopped. In the same conditions, the blank was prepared by adding methanol solution to all the reaction reagents without enzyme (α-glucosidase) in solution. The control was prepared by adding solvent extract to all reagents with the enzyme. The blank and the control absorbances were measured by spectrophotometer at 405 nm at 37°C each 10 min, over a period of 30 min. The absorbance of the blank was subtracted from that obtained with sample and the inhibitory activity of α-glucosidase was expressed as percentage in reference to acarbose and calculated following the formula:

$$\% \text{ Inhibition} = [(A_C - A_S) / A_C] \times 100$$

Where A_C is the absorbance of the control (100 % enzyme activity) and A_S is the absorbance of the tested sample (plant extract or acarbose).

8. Antimicrobial activity

The microorganisms used in the experiment were Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 25922) and fungi (*Fusarium oxysporum*). The latter was isolated and identified in the laboratory of Mycology at the Biotechnology research centre CRBt of Constantine, Algeria. Mueller-Hinton agar medium offered by Pasteur Institute of Algiers (Algeria) was used for bacteria, and PDA medium was used for fungi.

This test was carried out according to the well diffusion method described previously (Magaldi et al. 2004; Valgas et al. 2007) at a concentration of 16 mg/ml. This method consists of cutting circular holes (wells) of 6 mm of diameter in the agar and adding thereon extract with known concentration. The extract diffuses radially, giving a circular zone of inhibition

on the surface of the agar seeded with the bacterial suspension. The activity for tested bacteria was determined by measuring the diameter of the growth inhibition zone (inhibition zone diameter) visible around the wells where a diameter more than 6 mm indicated growth inhibition while 6 mm means no activity. For the antifungal test, the mycelia growth of phytopathogenic agents is measured on a millimeter scale. The results were expressed as a percentage of growth inhibition for each extract, relative to the average diameter of the colonies of fungi cultivated in a control medium. Thus, the inhibition activity was expressed as a percentage and calculated according to the following formula:

$$\% \text{ Inhibition} = [(C - T) / C] \times 100$$

Where C is the radical growth of the pathogen, in mm, on PDA medium with DMSO (control) and T is the radical growth, in mm, of the pathogen.

9. Statistical analyses

All the experimental results are mentioned as a mean ± standard deviation of three trials.

References

Apak R, Guçlu K, Ozyurek M, Karademir SE. 2004. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J Agric Food Chem. 52:7970–81.

Blois MS. 1958. Antioxidant determinations by the use of a stable free radical. Nature 26:1199-200.

Bothon FT, Debiton E, Avlessi F, Forestier C, Teulade J-C, Sohounhloue DK. 2013. *In vitro* biological effects of two anti-diabetic medicinal plants used in Benin as folk medicine. BMC Complement Altern Med. 13:51.

Hanamura T, Hagiwara T, Kawagishi H. 2005. Structural and functional characterization of polyphenols isolated from acerola (*Malpighia emarginata* DC.) fruit. Biosci Biotechnol Biochem. 69:280–286.

Kim JS, Kwon CS, Sou KH. 2000. Inhibition of alpha glucosidase and amylase luteolin, a flavonoid. BioscI Biotechnol Biochem. 64: 2458-2461.

Laoufi H, Benariba L, Adjdir S, Djaziri R. 2017. *In vitro* α-amylase and α-glucosidase inhibitory activity of *Ononis angustissima* extracts. J Appl Pharm Sci. 7: 191-198.

Magaldi S, Mata-Essayag S, De Capriles CH, Perez C, Colella MT, Olaizola C, Ontiveros Y. 2004. Well diffusion for antifungal susceptibility testing. Int J Infect Dis. 8*:* 39-45.

Randhir R and Shetty K. 2007. Improved α-amylase and *Helicobacter pylori* inhibition by fenugreek extracts derived via solid-state bioconversion using *Rhizopus oligosporus*. Asia Pac J Clin Nutr. 16: 382-392.

Sinéad L, Smyth TJ, Soler-Vila A, Stanton C, PaulRoss R. 2013. The alpha-amylase and alpha-glucosidase inhibitory effects of Irish seaweed extracts. Food Chem. 141: 2170–2176.

Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299:152-78.

Süntar İ, Baldemir A, Coşkun M, Keleş H, Akkol EK. 2011. Wound healing acceleration effect of endemic *Ononis* species growing in Turkey. J Ethnopharmacol. 135:63–70.

Tadera K, Minami Y, Takanatsu K, Matsuoka T. 2006. Inhibition of α-glucosidase and α-amylase by flavonoids. J Nutr Sci Vitamol. 52: 99-103.

Tel G, Apaydin M, Duru ME, Öztürk M. 2012. Antioxidant and cholinesterase inhibition activities of three *Tricholoma* species with total phenolic and flavonoid contents: The edible Mushrooms from Anatolia. Food Anal Methods. 5:495-504.

Topçu G, Ay A, Bilici A, Sarıkürkcü C, Öztürk M, Ulubelen A. 2007. A new flavone from antioxidant extracts of *Pistacia terebinthus*. Food Chem. 103: 816–822.

Valgas C, Souza SMD, Smânia EFA, Smânia Jr A. 2007. Screening methods to determine antibacterial activity of natural products. Braz J Microbiol. 38: 369–380.

Williams G. 2013. Possible effects of datary polyphenols on sugar absorption and digestion. Mol Nutr Food Res. 57:48-57.

Xiao J, Huo J, Jiang H, Yang F. 2011. Chemical compositions and bioactivities of crude polysaccharides from tea leaves beyond their useful date. Int J Biol Macromol. 49: 1143–1151.

Zengin G, Sarikurkcu C, Aktumsek A, Ceylan R, Ceylan O. 2014. A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Ind Crops Prod. 53: 244-251.

Table S1. Chemical composition of *O. alba* essential oil.

			% essential oil
Component	**RI calc.**	**RI lit.**	
(2*E*)-Hexenal	847	846	0.2
(3*Z*)-Hexenol	848	850	0.1
(2*E*,4*E*)-Hexadienal	909	907	0.2
2-Pentylfuran	988	984	0.1
Octanal	1003	998	0.1
Limonene	1024	1024	0.2
Octanol	1072	1063	0.1
Fenchone	1083	1083	0.1
2-Nonanone	1093	1087	0.1
Nonanal	1104	1100	0.5
(2*E*,6*Z*)-Nonadienal	1153	1150	0.1
2*E*-Nonen-1-al	1159	1157	0.1
Nonanol	1173	1165	0.2
Nonanenitrile	1184	1182	0.1
Dihydrocarveol	1191	1192	0.1
2-Decanone	1193	1190	0.8
Decanal	1205	1201	0.3
β-Cyclocitral	1215	1217	0.1
Cuminaldehyde	1234	1238	0.8
Carvone	1239	1239	0.4
Bornyl acetate	1280	1287	0.1
Nonanoic acid	1288	1267	0.5
2-Undecanone	1296	1293	30.5
Carvacrol	1303	1298	0.1
Undecanal	1306	1305	0.2
(2*E*,4*E*)-Decadienal	1314	1315	0.1
Methyl decanoate + iso-Dihydrocarveol acetate	1325	1323, 1326	0.2
1,2-Dihydro-1,1,6-trimethylnaphthalene	1341	1344	0.1
Eugenol	1353	1356	0.3
(*Z*)-Carvyl acetate	1360	1365	0.1
β-Bourbonene	1374	1387	0.5
(*E*)-β-Damascenone	1378	1383	1.6
Decanoic acid	1397	1363	5.5
2-Dodecanone	1393	1395	2.1
1-Dodecyn-4-ol	1398	-	0.2

(*E*)-β-Damascone + (*E*)-Caryophyllene	1406	1413; 1417	0.4
β-Copaene	1416	1430	0.1
Geranyl acetone	1449	1453	0.3
Undecanoic acid	1473	1475	0.5
(*E*)-β-Ionone	1476	1487	0.7
Dodecane nitrile	1483	1490	0.2
2-Tridecanone	1491	1495	9.7
Tridecanal	1503	1509	0.1
Methyl dodecanoate	1519	1524	0.1
Spathulenol	1562	1577	0.1
Caryophyllene oxide	1566	1582	0.4
Dodecanoic acid	1574	1565	3.0
Tridecane nitrile	1585	1592	0.1
2-Tetradecanone	1592	1595	0.9
β-Himachalene oxide	1596	1615	0.2
Caryophylla-4(12),8(13)-dien-5α-ol	1621	1639	0.1
epi-α-Cadinol	1625	1638	0.3
epi-α-Muurolol	1627	1640	0.1
δ-Cadinol	1634	1653	0.1
α-Cadinol	1641	1652	0.6
(*E*)-Calamenen-10-ol	1646	1660	0.1
Tridecanoic acid	1666	1666	0.3
2-Pentadecanone	1694	1700	1.0
Methyltetradecanoate	1722	1722	0.1
(*E*)-2-Hexyl cinnamaldehyde	1737	1748	0.1
Phenanthrene	1748	1752	0.1
Tetradecanoic acid	1770	1768	2.5
Hexahydrofarnesyl acetone	1842	1846	1.8
1-Phenyl-1-decanone	1872	1903	0.3
6-Nonyl-5,6-dihydro-2H-pyran-2-one	1901	1910	0.1
2-Heptadecanone	1898	1907	0.2
(5*E*,9*E*)-Farnesyl acetone	1913	1913	0.4
Methyl hexadecanoate	1925	1921	0.2
Phytol	1945	1942	0.1
Carbonic acid, 2-ethylhexyl nonyl ester	1963	1953	1.3
Hexadecanoic acid	1970	1968	3.1
Eicosane	1997	2000	0.2
Methyl 9,12-octadecadienoate	2084	2101	0.1

Methyl linolenate	2089	2108	0.4
Heneicosane	2089	2100	0.1
(*E*)-Phytol	2103	2113	4.5
Linoleic acid	2118	2132	0.3
Linolenic acid	2135	2154	0.8
3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	2218	2232	0.1
Tricosane	2300	2300	0.2
Tetracosane	2400	2400	0.1
Heptacosane	2700	2700	0.3
Nonacosane	2900	2900	0.3
Alcohols			0.5
Aldehydes			1.8
Alkanes			1.1
Ketones			45.1
Carboxylic acids			17.9
Esters			2.9
Terpenoids			9.3
OTHERS			4.9
Number of identified compound			85
Total % of identified compound			83.5

Table S2. Antimicrobial activity (inhibition zone and percentage for bacteria and fungi, respectively) of *O. alba* extracts.

Extracts (16 mg/ml)	Microorganisms		
	S. aureus ATCC25923	*E. coli* ATCC 25922	*F. oxysporum*
ethyl acetate	-	-	3.78%
dichloromethane	13 mm	-	9.84%
n-butanol	10 mm	-	6.81%

Table S3. IC_{50} values for the inhibitory properties of *O. alba* extracts against α-amylase and α-glucosidase enzymes.

Extracts	**IC_{50} (µg/ml)**

	α-amylase	**α-glucosidase**
ethyl acetate	17.80 ± 4.32	NA
dichloromethane	20.24 ± 0.3	NA
n-butanol	< 6.25	NA
acarbose	365.09 ± 10.7	275.43 ± 1.59

NA: not active.