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Screening for acetylcholinesterase inhibition, lipid peroxidation inhibition and antioxidant activity of medicinal plants from Morocco

[Detección de inhibición de acetilcolinesterasa, inhibición de peroxidación de lípidos y actividad antioxidante de plantas medicinales de Marruecos]

Smail Aazza[1], Soukaina El-Guendouz[2] & Maria da Graça Miguel[2]

[1]Laboratory of Phytochemistry, National Agency of Medicinal and Aromatic Plants (NAMAP/ANPMA) - Taounate, Morocco.
[2]Department of Chemistry and Pharmacy, Mediterranean Institute for Agriculture Environment and Development, Faculty of Science and Technology University of Algarve, Campus de Gambelas, Faro, Portugal

Reviewed by:
Marcelo L. Wagner
Universidad de Buenos Aires
Argentina

Ivan Montenegro
Universidad de Valparaíso
Chile

Correspondence:
Maria da Graça **MIGUEL**:
mgmiguel@ualg.pt

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Abstract: Acetylcholinesterase (AChE), hydrolyzes acetylcholine to choline and acetate, thereby terminating this neurotransmitter effect at cholinergic synapses. Therefore, AChE inhibition is used for counterbalance the cholinergic deficit in Alzheimer's disease (AD) patients. In the present work, in order to find new plant acetylcholinesterase inhibitors, the hydroalcoholic extracts from seventeen medicinal plant species were screened for their acetylcholinesterase inhibition activity, as well as total phenolic (TPC) and flavonoids contents (TFC) and antioxidant activity using ORAC (Oxygen Radical Absorbance Capacity) assay, and their ability to inhibit lipid peroxidation. The results revealed that *Rumex acetosa*, *Taraxacum officinale* and *Hypericum perforatum* extracts possessing the highest TPC and TFC, were the most effective in terms of ORAC antioxidant activity, and acetylcholinesterase inhibition, in addition to their ability to inhibit liposomes peroxidation, suggesting that those plant species may provide a substantial source of secondary metabolites, which act as natural antioxidants and acetylcholinesterase inhibitors, and may be beneficial in the treatment of AD.

Keywords: Alzheimer; Liposome peroxidation; ORAC; *Rumex acetosa*; *Taraxacum officinale*; *Hypericum perforatum*.

Resumen: La acetilcolinesterasa (AChE) hidroliza la acetilcolina se hidroliza en colina y acetato, terminando así este efecto neurotransmisor en las sinapsis colinérgicas. Por lo tanto, la inhibición de la AChE se utiliza para contrarrestar el déficit colinérgico en pacientes con enfermedad de Alzheimer (EA). En el presente trabajo, con el fin de encontrar nuevos inhibidores de la acetilcolinesterasa vegetal, se analizaron los extractos hidroalcohólicos de diecisiete especies de plantas medicinales para determinar su actividad inhibidora de la acetilcolinesterasa, así como el contenido total de fenólicos (TPC) y flavonoides (TFC) y la actividad antioxidante utilizando ORAC (Capacidad de absorbancia de radicales de oxígeno) y su capacidad para inhibir la peroxidación de lípidos. Los resultados revelaron que los extractos de *Rumex acetosa*, *Taraxacum officinale* e *Hypericum perforatum* que poseen los más altos TPC y TFC, fueron los más efectivos en términos de actividad antioxidante ORAC e inhibición de acetilcolinesterasa, además de su capacidad para inhibir la peroxidación de los liposomas, sugiriendo que esas especies de plantas puede proporcionar una fuente sustancial de metabolitos secundarios, que actúan como antioxidantes naturales e inhibidores de la acetilcolinesterasa, y puede ser beneficioso en el tratamiento de la EA.

Palabras clave: Alzheimer; Peroxidación de liposomas; ORAC; *Rumex acetosa*; *Taraxacum officinale*; *Hypericum perforatum*.

INTRODUCTION

Alzheimer's Disease (AD) is a progressive irreversible neurodegenerative disorder associated with oxidative stress and characterised by excessive deposition of β-amyloid (Aβ) oligomers, and neurofibrillary tangles (NFTs), comprising of hyperphosphorylated tau proteins (Chiorcea-Paquim *et al*., 2020). Moreover, an insidious decline in cognitive and non-cognitive functions is observed which are the most common single causes of dementia in our aging society. These impaired functions have been attributed to a loss of cholinergic neurons (McGleenon *et al*., 2001) and acetylcholine (ACh) neurotransmitter hypofunction in the brain (Patel *et al*., 2018). Although, it is estimated to be responsible for 50-60% of dementia cases in persons over 65 years-old (Barbosa Filho *et al*., 2006), which incidence is doubling almost every 5 years from the ages 65 to 90 years-old (Corrada *et al*., 2010).

Acetylcholinesterase (AChE), the predominant cholinesterase in the brain, is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter ACh in numerous cholinergic pathways in the central and peripheral nervous systems (Lazarevic-Pasti *et al*., 2017). It is well established that the AChE is associated with the pathogenesis and progression of AD. Hence, inhibiting this enzyme activity is one of the promising approaches for treating some symptoms of this disease, by enhancing the ACh level in the brain (Barbosa Filho *et al*., 2006). Consequently, AChE inhibitors (AChEi) or anticholinesterases, which represents an important therapeutic strategy in Alzheimer's disease, are the main class of drugs currently used for the treatment of AD dementia phase, leading to an increasing of both the level and duration of the neurotransmitter action (Colovic *et al*., 2013) and improving cholinergic function in the brain (Andrieu *et al*., 2015). Clinically relevant AChEi, such as donepezil, rivastigmine, and galantamine, are commonly applied in neurodegenerative disorders treatment, especially in the pharmacotherapy of Alzheimer's disease (Colovic *et al*., 2013). Within those clinically relevant AChEi, galantamine is of natural origin being an alkaloid extracted from species of the Amaryllidaceae family (Roseiro *et al*., 2012; Murray *et al*., 2013). However, the management of the diseases using the current drugs is accompanied by various drawbacks (Adewusi *et al*., 2011).

Furthermore, increased lipid peroxidation and elevated oxidative stress also represent well-established characteristics of AD (Kontush, 2006). Among all AD hallmarks, oxidative damage has been reported as the first observable event in the disease progression (Persson *et al*., 2014). Inasmuch as, the central nervous system (CNS) remains one of the major targets of lipid peroxidation leading to the formation of highly reactive electrophilic aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein, which is the most reactive (Sultana *et al*., 2013). Thus, efforts to reduce the pathology associated with reactive oxygen species (ROS) *via* antioxidants offer new hope to patients suffering from this devastative disease (Aliev *et al.*, 2008). Subsequently, searching for natural products acting as antioxidants and acetylcholinesterase and lipid peroxidation inhibitors will be of paramount importance. Numerous studies have been realized several plant species in order to identify and isolate naturally occurring phytochemicals diverse classes of alkaloids, coumarins, terpenes, and polyphenols, which can be applied for new anti-AD drugs development (dos Santos *et al.*, 2018).

The genus *Berberis* is widespread all over the world, from India, Pakistan, Japan, China, to Europe, Africa, South America and North America (Khan *et al*., 2016). This genus consists of 594 species worldwide and *Berberis vulgaris* L. and *B. integrimma* have been used in the islamic world for the treatment of skin, liver, stomach, kidney, and eye problems (Khan *et al*., 2016; Sobhani *et al*., 2021). Aqueous root extract of *B. vulgaris* was also reported as presenting significant hypoglycemic effects in streptozotocin-induced diabetic rats (Khan *et al*., 2016).

Centaurium erythraea Rafn. is widely distributed in the Mediterranean region used in the treatment of digestive, renal, hepatic, respiratory, cardiovascular, namely hypertension, and rheumatic diseases, and antidiabetic agent. Other studies have been noticed the antipasmodic, antioxidant, diuretic, and gastroprotective properties (Chda *et al*., 2020).

Ceratonia siliqua L. (carob) is widely distributed in the Mediterranean region, namely Italy, Portugal, Morocco, Greece, Cyprus, Turkey, and Algeria, which are important producers of carob worldwide. Carob is mainly used in food industries in order to produce locust bean and carob bean gum for cosmetics and textile. *C. siliqua* has been reported as possessing antimutagenic, antiproliferative, antiestrogenic, nephroprotective and anthelmintic properties. It has also been attributed to this species antioxidant activity and ability to prevent

cardiovascular disease, reduce hypercholesterolemia in humans, and inhibit acetylcholinesterase activity (Abidar *et al*., 2019).

Glycyrrhiza glabra L. is native to the Mediterranean areas but it can also be found in India, Russia and China. This species is important in food industry as flavours and sweetening agents. In beers and fire extinguishers, the root extracts are used as foaming agents. Since ancient times this species has been used in folk medicine in the treatment of gastrointestinal problems, cough, bronchitis, and arthritis and tremors. A review made by Pastorino *et al*. (2018) is compiled the biological properties that have been studied. They are antioxidant, antiinflammatory, antitussic and expectorant, anti-ulcerative, antimicrobial, antiviral, anticarcinogenic and antimutagenic, hepatoprotective, neuroprotective, sedative anti-depressive, osteogenic and androgenic, among other properties (Pastorino *et al*., 2018). Reciently, it has been shown that the extract of *Glycyrrhiza glabra* root attenuates nociception in experimental pain models (Parlar *et al*., 2022). The good antioxidant activity and the inhibitory activity on urease made Lateef *et al*. (2012), consider this species as a good possibility in the ulcer treatment.

The present study suggests that roots of *Glycyrrhiza glabra* is a potential source of antioxidants and urease inhibitors and the constituents may considered as a lead compound in the study of drug discovery and designing for ulcer treatment.

Crataegus monogyna Jacq. grows in Europe, Africa, and Asia. Several pharmacological properties have been attributed to this species, such as neuroprotective, hepatoprotective, cardioprotective, with capacity for reducing some cardiovascular risk factors, such as hypertension, hypercholesterolaemia, also possessing tonic effects on the heart, and nephroprotective (Nabavi *et al*., 2015).

Cynara scolymus L. is a native species of the Mediterranean basin but cultivated all over the world for edible and medicinal purposes. This species has been reported as possessing antimicrobial, hepatoprotective, cholerectic, hypocholesterlomic, hypoglycemic, antioxidant and anticancer properties (Salekzamani *et al*., 2019).

Silybum marianum (L.) Gaertn. is originated in the Mediterranean Basin, but grows in many European countries, North Africa, South and North America, Central and Western Asia and southern Australia (Valková *et al*., 2020). Antimicrobial, anticancer, hepatoprotective, cardioprotective, neuroprotective, skin-protective, antidiabetic and detoxification effects are some attributed to this species and reviewed by Wang *et al*. (2020).

Marrubium vulgare L. Herb. grows in the Mediterranean areas of Europe and North Africa and it has been used for antihypertensive therapy, antispasmodic therapy for acute or chronic bronchitis and colds, expectorant and in cases of asthma, appetite loss and dyspepsia (Villanueva & Esteban, 2016). The review made by these authors, they report many pharmacological actions, such as antiinflammatory and analgesic, antisapsmodic and vasorelaxant, gastroprotective, hepatoprotective, antidiabetic and hypocholesterolemic, antihypertensive, antioxidant, antimicrobial and antifungal, antiasthmatic and expectorant and neuroprotective. However, the European Medicines Agency (EMA) describes the traditional use of this species as expectorant in catarrh associated with cooling (Villanueva & Esteban, 2016).

Melissa officinalis L. originated in Southern Europe, is currently naturalized around the world, from North America to New Zealand (Miraj *et al*., 2017). According to the review made by Shakeri *et al*. (2016), the authors compiled the pharmacological studies found for *M. officinalis* that possessed the following biological activities: antioxidant, hypoglycemic, hypolipidemic, antimicrobial, anticancer, antidepressant, anxiolytic, antinociceptive, anti-inflammatory and spasmolytic properties, although the review made by Miraj *et al*. (2017), also reported hypotensive, memory-enhancing, menstrual-inducing, and thyroid-related effects; antiparasitic, and in the treatment of flatulence, asthma, bronchitis, amenorrhea, cardiac failure, arrhythmias, ulcers, wounds, headaches, indigestion, colic, nausea, anemia, vertigo, syncope, malaise, and insomnia.

Salvia officinalis L. can be found in Europe around the Mediterranean, in Southeast Asia, and Central and South America. Anti-inflammatory and antinociceptive effects, antioxidant and antidementia properties related to Alzheimer's disease, antimicrobial and antiparasitic properties, anticancer and antimutagenic, and hypoglycemic and hypolipidemic effects were reported in a review on *S. officinalis* made by Jakovljević *et al*. (2019).

Fraxinus excelsior L. is widespread and can be found in France, north American, north east Asia, China, north Pakistan, India, Afghanistan, Morocco, and Algeria. Anticancer, antiinflammatory, neuroprotective, antioxidant, anticytotoxic, antiaging,

antimicrobial, and antihypertensive are some effects reported by Sarfraz *et al*. (2017).

Hypericum perforatum L. is native Europe, western Asia, and northern Africa, although currently it has a worldwide distribution. *H. perforatum* is a species largely known for its antidepressant-like properties, nevertheless other proprieties have been reported such as neuroprotective, nootropic, antimicrobial, antiviral and antiprotozoal, antiepileptic, anxiolytic, antitumor and cytotoxic properties, analgesic, antiinflammatory and wound-healing properties, hypolipidaemic and hypoglycaemic properties, among other attributes (Oliveira *et al*., 2016; Budantsev *et al*., 2021). According to the Kraus *et al*. (2007), the plant extract of *H. perforatum* was able to restore or improve microglial viability and thereby attenuate amyloid-β mediated toxicity in Alzheimer's disease, even better than individual flavonoids.

Passiflora edulis Sims., is originated in North America. This species is largely used in phytotherapy as a mild sedative and anxiolytic, nevertheless this species has been also target of study in the treatment of diverse ailments: treatment of opiate withdrawal, menopausal symptoms, insomnia/sleep disorders, and treatment of attention deficit hyperactivity disorder (ADHD) (Miroddi *et al*., 2013).

Phoenix dactylifera L. is cultivated for the production of dates that are been used for dietary, nevertheless diverse biological properties (antioxidant, antimicrobial, anticancer, antimutagenic, antiinflammatory, hypoglycemic, and sperm quality improvement activities), which may permit to use it to treat several diseases (Qadir *et al.*, 2020). Many countries of Middle East, North Africa, Sudan, Oman, Central and South America, Southern Europe, and some parts of India and Pakistan cultivate this species for the production of dates (Qadir *et al*., 2020).

Rumex acetosa L. is used as sauces and as a spinach or salad leaf. The sap is employed to remove stain while washing clothes. This species is cultivated in Europe and North America. *R. acetosa* shows medicinal importance due to their pharmacological properties: antimutagenic and antigenotoxic activity, antiperiodontitis, antiproliferative, antimicrobial, and antioxidant activities, to treat stomach discomfort and distress, and against gastritis and gastric ulcers (Bello *et al*., 2019).

Ziziphus lotus L. (Lam) is widely distributed in Mediterranean region, like Algeria, Morocco, Tunisia, and Libya. This plant is known for several medicinal applications as antidiabetic (Jouad *et al*., 2001; El-Hilaly *et al*., 2003; Hseini & Kahouadji, 2007; Abouri *et al*., 2012; Bouayyadi *et al*., 2015; Ouhaddou *et al*., 2015; Barkaoui *et al*., 2017; Marmouzi *et al*., 2019), sedative, bronchitis, heart disease, circulatory and urinary affections, digestive system, gastrointestinal disorders and antidiarrhea, hair care, against rheumatism and cold by local populations (El-Hilaly *et al*., 2003; Hseini *et al*., 2007; Abouri *et al*., 2012; Bakhtaoui *et al*., 2014; Bouayyadi *et al*., 2015; Ouhaddou *et al*., 2015; Benali *et al*., 2017; Marmouzi *et al*., 2019).

In order to search new phytochemical sources for the treatment of AD, seventeen medicinal plants used in the Moroccan Pharmacopeia, namely *Berberis vulgaris* L. (Berberidaceae); *Centaurium erythraea* Rafn. (Gentianaceae); *Ceratonia siliqua* L. and *Glycyrrhiza glabra* L. (Fabaceae); *Crataegus monogyna* Jacq. (Rosaceae); *Cynara scolymus* L., *Silybum marianum* (L.) Gaertn., and *Taraxacum campylodes* G.E. Haglund (syn. *Taraxacum officinale* (L.) Weber ex F.H. Wigg.) (Asteraceae); *Marrubium vulgare* L., *Melissa officinalis* L., and *Salvia officinalis* L. (Lamiaceae); *Fraxinus excelsior* L. (Oleaceae); *Hypericum perforatum* L. (Hypericaceae); *Passiflora edulis* Sims. (Passifloraceae); *Phoenix dactylifera* L. (Arecaceae); *Rumex acetosa* L. (Polygonaceae); and *Ziziphus lotus* (L.) Lam. (Rhamnaceae) were evaluated for their AChE and lipid peroxidation inhibition capacity.

MATERIAL AND METHODS

Plant material

Fruits from *C. siliqua*, *F. excelsior, B. vulgaris, Z. lotus, C. monogyna, F. excelsior*, and *B. vulgaris;* leaves from *M. vulgare, R. acetosa, P. edulis, R. acetosa*, *S. marianum* and *T. officinale;* male flowers pollen from *P. dactylifera*, stem from G. *glabra and the* aerial part of *C. erythraea*, *H. perforatum, and M. officinalis* have been purchased from herbalists in the region of Fez, Morocco. Subsequently, purchased samples were ground and subjected to extraction.

Plant solvent extraction

The extraction of phenols was performed by sonication on an ice-bath for 6 min using a VC300 Vibracell sonicator (Sonics and Materials, USA) with a 20 kHz frequency. One gram of dried powder in 10 mL of a hydro-alcoholic solution (70%) was used. After sonication, the samples were centrifuged for 5 min at 2,000 *g* at 20°C, and the supernatant was removed and kept at -20ºC for successive analyses.

Total phenolic content (TPC)

The total phenolic content was determined using Folin-Ciocalteu reagent according to the method described by Singleton & Rossi (1965), with some modifications to adapt to 96 well microplate. Plant hydro-alcoholic extracts (25 µL) were mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3, and the absorbance was measured at 760 nm after 2 h of incubation at room temperature. The total polyphenol content was expressed as mg per g of Gallic Acid Equivalents (GAE) using a calibration curve.

Total flavonoid content (TFC)

The amounts of flavones and flavonols in extracts were determined according to the method described by Miguel *et al*. (2010), with minor modifications. An amount of 100 µL of $AlCl_3$ (20%) was added to 100 µL of extract, and after 1 h at room temperature, the absorbance was measured at 420 nm. Total flavones and the flavonols content were calculated as Quercetin Equivalents (QE) mg per g, using a calibration curve.

Oxygen radical activity capacity (ORAC) assay

The ORAC method used fluorescein (FL) as the "fluorescent probe" and was performed according to the method described by Ou *et al*. (2001). This assay is based on the capacity of antioxidants in a sample to quench peroxyl radicals that are generated from the thermal decomposition of AAPH (α,α'-Azodiisobutyramidine dihydrochloride). AAPH (0.414 g) was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM and was kept in an ice bath. The fresh fluorescein working solution (8.16×10^{-5} mM) was made daily in 75 mM phosphate buffer (pH 7.4). Trolox standard curve was prepared in the phosphate buffer with the following concentrations: 50, 25, 12.5, and 6.25 µM. In each well (96 well TC treated plate, black, µclear, Greiner BioOne) 150 µL of fluorescein working solution and 25 µL of the sample, blank (75 mM phosphate buffer), or standard (Trolox) were placed. The plate was covered with a lid and incubated in the preheated (37ºC) BioTek Synergy™ 4 Hybrid Microplate Reader for 10 min with a previous shaking of 3 min. AAPH was added to each well of the plate, except for the control and blank. The final volume of the assay was 200 µL. The microplate was shaken for 10 s, and fluorescence was read every minute for 90 min at excitation of 485 nm and emission of 527 nm. ORAC values were calculated by calculating the net area under the curve (AUC) of the standards and samples. The standard curve is obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC and are expressed as micromole Trolox equivalents per liter of samples.

Inhibition of lipid peroxidation of lecithin liposomes

Lipid peroxidation (LP) was measured according to the method described by Aazza *et al*. (2014). Liposomes were obtained from 0.4 g lecithin in 80 mL chloroform. This solution was dried under vacuum in a rotary evaporator (<50ºC) to yield a thin, homogenous film and after submitted to nitrogen flux for 30 s. Liposomes were then submitted to vacuum for at least two hours until complete dryness. The film was then dispersed in 80 mL of phosphate saline buffer 0.01M, pH 7.0 (Fisher, Bioreagents, NJ, USA). The mixture was sonicated to obtain a homogeneous suspension of liposome and kept at 4ºC until the assay.

The extent of LP was determined by measuring the color of the adduct produced in the reaction between TBA (thiobarbituric acid) and malondialdehyde (MDA), as an oxidation product in the peroxidation of membrane lipids, by the TBA assay. Different concentrations of samples were prepared in ethanol. A control with ethanol instead of the sample was also analyzed for the system of induction of LP. The reaction mixture Fe^{2+}/ascorbate-induced LP, 60 µL of a suspension of liposomes was incubated with 20 µL of 0.01 M $FeSO_4$, 20 µL of 0.01 M ascorbic acid, and 10 µL of samples in 2.89 mL of 0.05 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4 (3 mL final solution).

Samples were incubated at 37ºC for 1 h. LP was terminated using the reaction with 1.5 mL of TBA reagent and 0.2 mL of 0.1 M EDTA, heating at 100ºC for 20 min. After the solution was cooled and the precipitated proteins were centrifuged (4,000 rpm for 10 min), the content of the MDA was determined by measuring the absorbance of the adduct at 532 nm.

All of the reactions were carried out in triplicate. The percentage of LP inhibition was calculated by the following equation:

$$\mathbf{I\ (\%) = [(A_o - A_1)/A_o] \times 100}$$

Where A_o was the absorbance of the control reaction

(full reaction, without the test compound) and A_1 was the absorbance in the presence of the inhibitor. The results were presented as IC_{50} (the concentration of the sample required to inhibit 50% of lipid peroxidation of lecithin liposomes), calculated by linear regression analysis

Acetyl cholinesterase (AChE) inhibition

AChE inhibitory activities of the extracts were determined by the modified Ellman spectrophotometric method with minor modifications as described by Aazza *et al*. (2011). Fifty µL of buffer 0.1 M (pH 8), 25 µL of plant extract at different concentrations and 25 µL of 0.22 U/mL of AChE enzyme were mixed. After 15 min incubation at 37°C, 25 µL of 15 mM acetylthiocholine iodide (AChI) and 125 µL of 3 mM 5,5'- dithiobis [2-nitrobenzoic acid] (DTNB) were added and the resulting mixture incubated for 30 min at room temperature. The absorbance of the mixture was measured at 405 nm by using a microplate reader. The inhibitory effect of the test compound was calculated by comparing to the negative control:

$$\%= [(A_0–A_1)/ A_0] \times 100$$

where A_0 was the absorbance of the blank sample and A_1 was the absorbance of the sample. The test was repeated three times. The inhibition of enzyme activity was expressed as IC_{50} (the concentration of the sample required to inhibit 50% of enzyme), calculated by linear regression analysis.

Statistical analysis

The data analysis was performed in triplicate. One-way ANOVA and Post-hoc Tukey's HSD test at 95% confidence limit, statistically significant at $p<0.05$ level, has been calculated to confirm statistically significant differences between samples using Graphpad Prism software (Trial version 8.3.1). Results have been correlated using regression analysis and statistically evaluated using analysis of variance (ANOVA). PAST 4.02 (Paleontological Statistics Software Package for Education and Data Analysis) packages was used to perform Principal Component Analysis (PCA) in order to identify relationships between the variables.

RESULTS AND DISCUSSIONS

Total phenolics content

Plant extracts possessing high total phenolic contents can be promising as ingredients of functional foods and nutraceuticals. Natural phenolic compounds occurring mainly as secondary metabolites in a wide variety of chemical structures, are ubiquitous in the vegetable kingdom and counts around 10,000 different plant phenolic derivatives (Roseiro *et al*., 2012). The neuroprotective effects of polyphenols are widely studied and documented (Ullah & Khan 2018). Several polyphenols have been reported to have a significant AChE inhibiting potential, by interacting with amino acid residues defining the active site of AChE via a hydrogen bond, hydrophobic, and π–π interaction, and this binding capacity is enhanced by the multiple hydroxyl groups in the phenolic compound (Jabir *et al*., 2018). The total phenolic content of the hydroalcoholic extracts from studied species was determined spectrophotometrically and expressed as mg gallic acid per g of the dry plant (or dry part used of the plant) (Figure No. 1). The results revealed a very wide variation among extracts, ranging from 0.45 ± 0.05 to 80.65 ± 0.41 mg GAE/g dw. Other authors reported similar patterns variation of TPC ranging between 1.27 mg and 117.20 mg GAE per g dw from 28 plant materials including *H. perforatum, S. officinalis* and *M. officinalis* (Kırca & Arslan, 2008). According to Figure No. 1, the highest TPC were found in *T. officinale*, followed by *R. acetosa* (77.90 ± 1.36 mg GAE/g dw), *H. perforatum* (76.07 ± 3.49 mg GAE/g dw) and *C. monogyna* (50.18 ± 0.98 mg GAE/g dw), whereas the lowest level was found in *C. scolymus.* Besides, *S. officinalis, M. officinalis, B. vulgaris, G. glabra, M. vulgare, S. marianum and C. erythraea* were also characterized by a high TPCs, which were above 20 mg GAE/g dw. *T. officinale* is a well-known medicinal plant to have various polyphenolic compounds, such as flavonoids and phenolic acids, which allows many attributed beneficial properties including anti-inflammatory, antioxidative, anti-hyperglycemic, and anticancer action (Miłek *et al*., 2019). *R. acetosa* is known to contain anthraquinones, tannins, flavonoids, and phenolic acids. Specimens collected from different locations in Serbia showed that ethanolic extracts from the aerial parts contain high amounts of polyphenols (Jovin *et al*., 2011). Compared to our findings; lower TPC amounts ranging from 3.23 to 9.84 mg GAE/g DW had been reported among *T. officinale* populations studied in different geographical regions from India, with an overall mean of 7.46 mg GAE/g dw (Singh Arya *et al*., 2015). Higher amount of TPC was reported for *H. perforatum* (84.70 ± 2.76 mg GAE/g), *S. officinalis*

(43.50 ± 1.34 mg GAE/g) and *M. officinalis* (69.20 ± 1.91 mg GAE/g dw) (Kırca & Arslan, 2008).

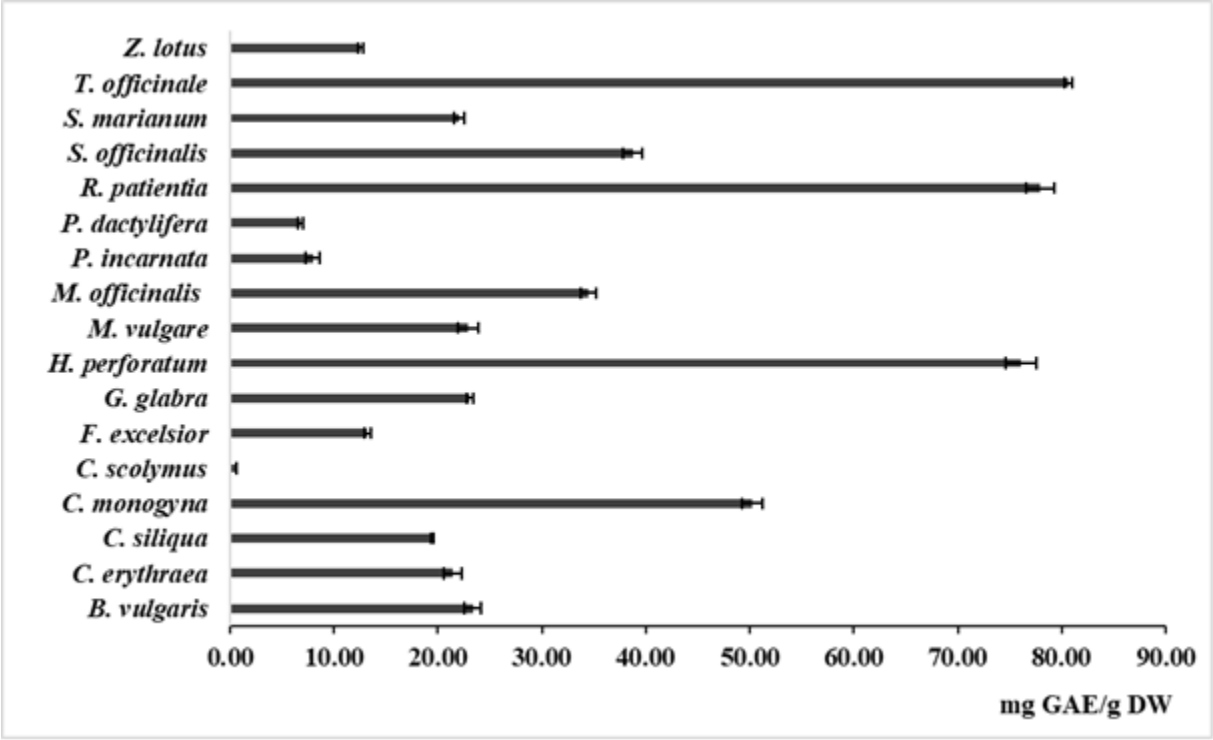

Figure No. 1
Total phenolic compounds (TPC) of the studied species (mg Galic Acid Equivalent (GAE) per g of plant dry weight)

Total flavonoids content

The beneficial effects of dietary flavonoids have been documented on a variety of neurological degenerative disorders including AD, in animal models, and several clinical trials (Khan *et al*., 2019). It has been demonstrated that the antioxidant and anti-inflammatory activities are involved in the neuroprotective effects of flavonoid compounds against neurological degenerative disorders, such as AD (Ullah & Khan, 2018). The results of TFC from the studied plant extract are depicted in Figure No. 2. In the screened plants hydroalcoholic extracts, TFC ranged from 0.19 to 15.30 mg QE/g dw, representing an approximate seventy-nine-fold variation. The differences in TFC among plant extracts used were statistically significant (p<0.05). *H. perforatum*, *R. acetosa* and *T. officinale* showed the greatest contents (15.30 ± 0.29, 12.86 ± 0.15 and 11.97 ± 0.09 mg QE/g dw, respectively), while the smallest contents, lower than 1 mg QE/g dw, were found in *C. scolymus, Z. lotus*, *S. marianum* and C. *silique* (0.19 ± 0.01, 0.21 ± 0.01 and 0.72 ± 0.03, 0.87 ± 0.02 mg QE/g dw, respectively). Furthermore, *G. glabra*, *C. erythraea*, *C. monogyna, B. vulgaris* present moderate amounts of TFC, above 5 mg QE/g dw. According to Silva *et al*. (2005), *H. perforatum* ethanolic extracts contained many phenolic compounds, namely flavonoids and phenolic acids, which were found to be the main contributors to its free radical-scavenging activity.

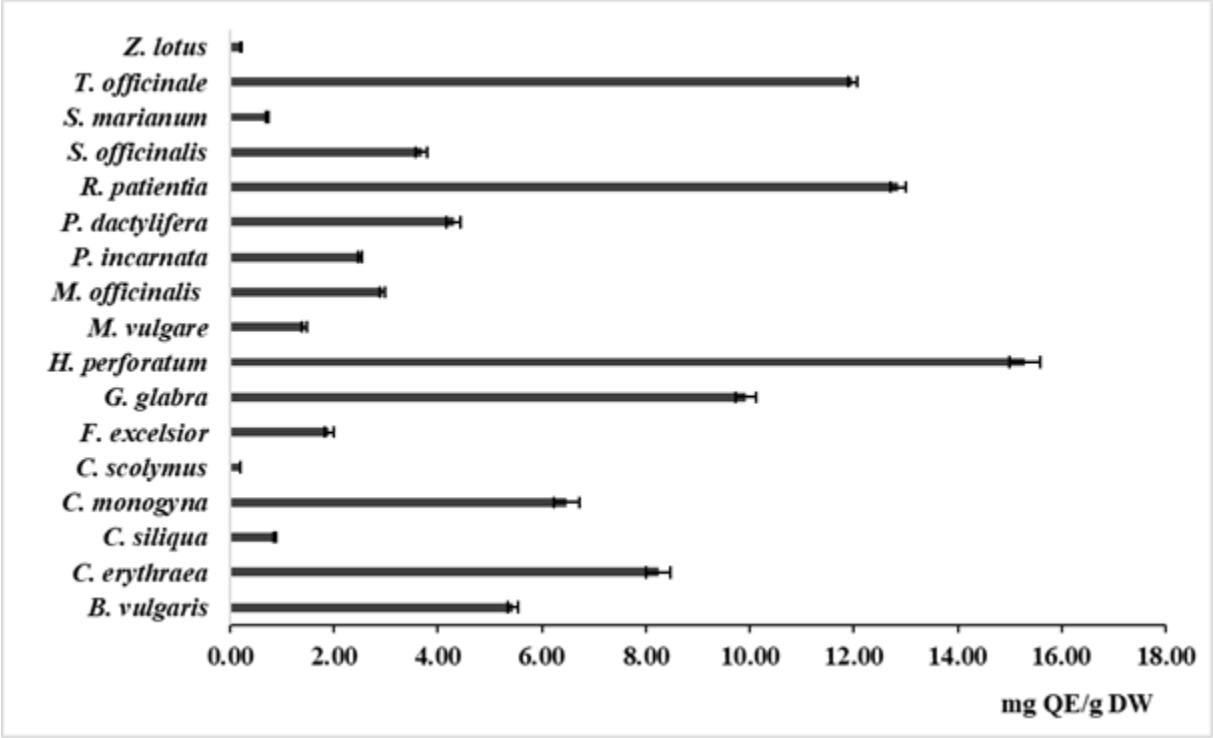

Figure No. 2
Total flavonoid content (TFC) of the studied species
(mg quercetin equivalent (QE) per g of plant dry weight)

Peroxyl radicals' scavenging activity

The age-related memory impairments were found to correlate with a decrease in brain and plasma antioxidants' defence mechanisms. Furthermore, glutathione concentration decreases with age mammalian brain regions including the hippocampus (Zhu *et al*., 2006). Hitherto, numerous evidences support the deteriorating action of free radicals, which can lead to cognitive aging and CNS (Ahmad *et al.*, 2015). Oxidative stress or the imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS) production and the intracellular capacity for removing them, is inevitably involved in the pathogenesis of AD, which subsequently leads to excessive damage of DNA, lipids, carbohydrates, and proteins in the cell (Vladimir-Knežević *et al*., 2014). Thereby, antioxidants can delay, inhibit, or prevent the oxidation biomolecules by scavenging free radicals and decreasing oxidative stress. The antioxidant capacity of the selected medicinal plant species was evaluated using oxygen radical absorbance capacity (ORAC) in which it is evaluated the ability of samples for scavenging peroxyl free radicals (inductors of neurodegenerative diseases), and the results are depicted in Figure No. 3. Results showed that all the tested extracts demonstrated antioxidant activity with a wide range of values among the studied species ranging from 12.97 to 830.78 µM TE/g, dw. The difference in antioxidant capacities was very large, up to 64-fold. The hydroalcoholic extract of *T. officinale* exhibited the most effective radical scavenging ability, followed by *R. acetosa* (744.57 ± 5.50 µM TE/g dw) and *H. perforatum* (667.28 ± 11.21 µM TE/g dw). The antioxidant activity of *T. officinale*, *R. acetosa* and *H. perforatum* was already previously reported (Mantle *et al*, 2000; Singh Arya *et al*., 2015; Zerrouki *et al*., 2016). Some other tested plants, such as *C. monogyna*, *S. officinalis, G. glabra, C. erythraea* and *B. vulgaris* presented good antioxidant activities ranging from 300 to 500 µM TE/g dw, whereas *C. scolymus*, *P. dactylifera* and *P. incarnate* presented the lowest ability for scavenging peroxyl free radicals. These three species were also characterized by the lowest phenolic content and antioxidant activity.

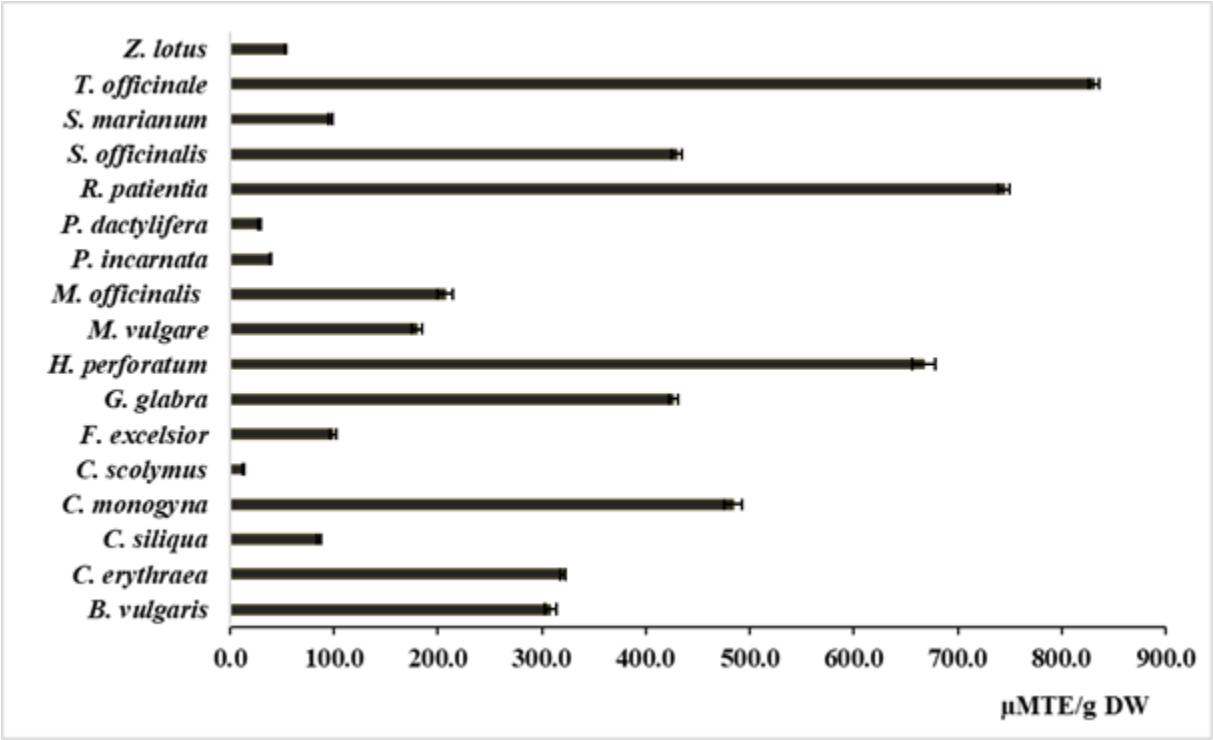

Figure No. 3
Oxygen radical absorbance capacity ORAC of the studied species expressed in µmol Trolox equivalent per g of plant dry weight

Lipid peroxidation inhibition (LPI)

Neural cells are considered to be more susceptible to oxidative damage as compared to other body tissues, as the brain is rich in peroxidizable fatty acids and has a high oxygen demand (Teixeira *et al*., 2013). It has been already shown that lipid peroxidation is a major cause of the depletion of membrane phospholipids in AD (Markesbery, 1997). In the present study, it was evaluated the extent of lipid peroxidation using the malondialdehyde (MDA) formation as an index of the breakdown of liposomes lipids and the results are shown in Figure No. 4. Most parts of plant extracts reduced lipid peroxidation in a dose-dependent manner and proved to be effective antioxidants. We noticed that extracts from *R. acetosa* (IC_{50}= 37.92 ± 0.51 µg/mL), *H. perforatum* (IC_{50} = 41.66 ± 1.30 µg/mL), *S. officinalis* (IC_{50} = 68.60 ± 1.21 µg/mL) and *T. officinale* (IC_{50} = 98.49 ± 6.67 µg/mL) were the most effective in protecting liposomes from lipid peroxidation. Moderate activity presenting an IC_{50} lower than 1 mg/mL was found in four species, namely *G. glabra*, *C. monogyna, C. siliqua,* and *B. vulgaris*, whereas *C. scolymus*, *P. incarnate* and *P. dactylifera* extracts did not show LPI higher than 50% in the range of the studied plant extracts concentrations.

Lipid peroxidation was significantly reduced in the presence of the *H. perforatum* ethanolic extracts and fractions containing flavonoids and/or caffeoylquinic acids. Additionally, flavonoid aglycones rich fractions were found to be responsible for a major part of the protection against lipid peroxidation. Furthermore, the bioflavonoid amentoflavone was found to be able to pass the blood-brain barrier by simple diffusion, making it plausible to suppose that the consumption of extracts of *H. perforatum* might be able to exert a positive action in the CNS (Silva *et al*., 2005). According to Oboh & Henle (2009), *S. officinalis* extracts inhibited MDA production in basal and pro-oxidant-induced lipid peroxidation in the brain and liver, besides their high antioxidant activity which could be harnessed in the management and prevention of degenerative diseases associated with oxidative stress. *T. officinale* extracts demonstrated an efficient ability to reduce lipid peroxidation generated by sodium nitroprusside (SNP) in the whole brain and brain structures, mainly attributed to the phenolic compounds present in the extract (Colle *et al*., 2012).

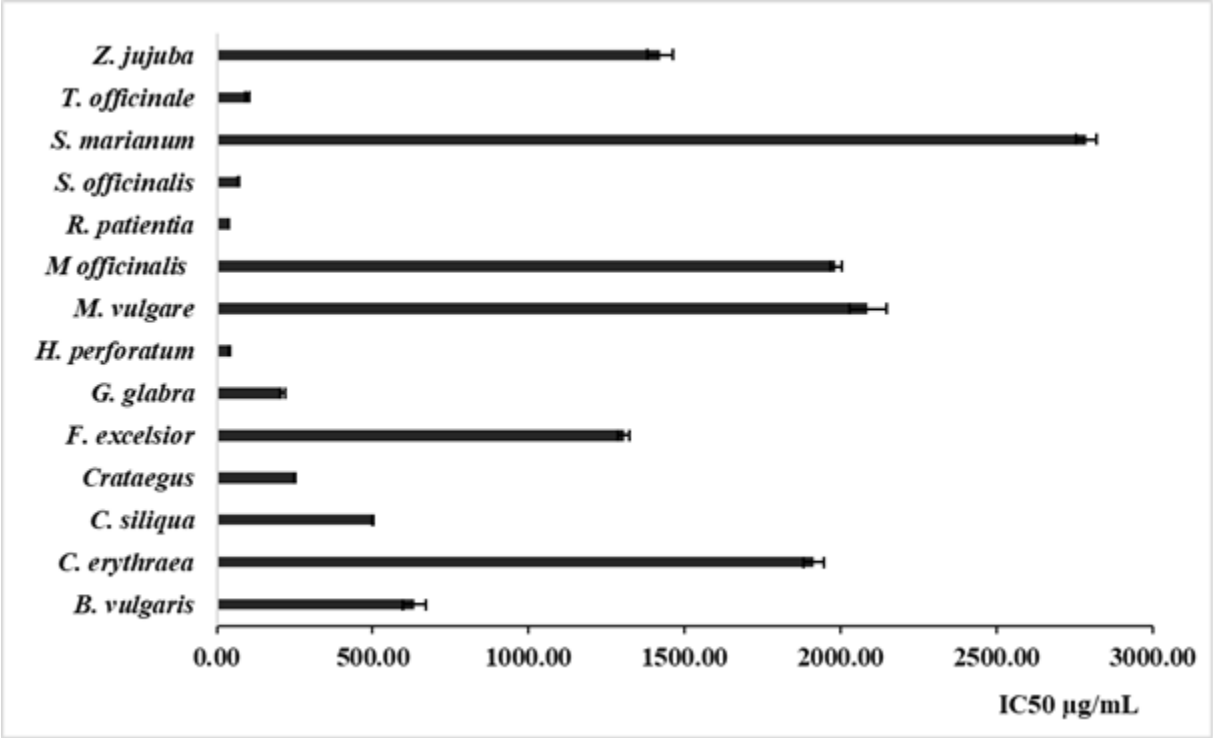

Figure No. 4
Liposomes peroxidation inhibition (LPI) of the studied species, results are expressed as (IC_{50} in mg/mL)

Acetylcholinesterase (ACh) inhibition

The decreased level of ACh within the nervous system may be due to a reduced level of AChE. Acetylcholine hydrolysis is decreased by inhibiting AChE in the brain (Ahmad *et al*., 2015). Acetylcholinesterase inhibitors are clinically used to treat several pathologies, including myasthenia gravis, glaucoma, Lewy body dementia, and AD, to improve symptoms through enhancing cholinergic functions and rising the amount of acetylcholine in cholinergic synapses (Roseiro *et al*., 2012). Inhibiting the AChE, as a means to increase the level of ACh, is one of the broadly recognized approaches for the treatment of AD (Urbain *et al*., 2004). According to the obtained results, all plant extracts were able to inhibit AChE activity in a dose-response manner (data not shown). The cholinesterase inhibitory activity of extracts is given in Figure No. 5 in IC_{50} values. AChE inhibition results showed a broad difference between plant extracts, and the IC_{50} was extending from 1.93 to 388.63 µg/mL. Based on their inhibition capacity towards AChE, the studied plant extracts could be ranked into three groups. The first groups represented by four plant species exhibiting high activity (IC_{50} <10 µg/mL), namely *R. acetosa*, *H. perforatum*, *T. officinale* and *M. vulgare*. The second group containing seven species with a good to medium inhibitory activity (10 µg/mL < IC_{50} < 50 µg/mL) and the last group exhibiting low activity which consist of six plant species (IC_{50} >50 µg/mL).

Among the studied species, extract from *R. acetosa* displayed the highest inhibition activity (IC_{50} = 1.93 ± 0.07 µg/mL), followed by both extracts of *T. officinale* (IC_{50} = 4.27 ± 0.2 µg/mL) and *H. perforatum* (IC_{50} = 4.32 ± 0.07 µg/mL) which were found to have the second-highest inhibition on AChE without any significant differences at $p > 0.05$. Moreover, *M. vulgare* showed the fourth highest activity (IC_{50}= 8.92 ± 0.11 µg/mL) followed by *C. erythraea* (IC_{50} = 13.11 ± 0.28 µg/mL) and *C. siliqua* (IC_{50} = 13.90 ± 0.11 µg/mL) without significant differences. This value is similar to that reported by Saci *et al*. (2020), for unrip carob pulp. The authors reported the importance of ripening stages on the ability for inhibitiong the AChE activity: riped pulp had lower activities. Leaves extracts have been also reported as being able to inhibit the AChE activity (Custódio *et al*., 2015; Abidar *et al*., 2019),

nonetheless much lower when compared to the results presented in the present work. On the other hand, we found that *S. marianum* and *P. dactylifera* extract were less effective in inhibiting AChE. Traditionally, *Rumex* species are used to treat neurological disorders comprising headache, migraine, depression, paralysis. Additionally, *R. acetosa* has been scientifically confirmed to improve memory and passive avoidance learning (Ahmad *et al*., 2015) and capacity for inhibiting acetylcholinesterase activity (Sarikurkcu *et al*., 2017). *H. perforatum* extracts containing hypericin as the main active ingredient, are used as one of the most useful natural antidepressant therapeutic agents in the treatment of mild to moderate depression (Spiridon *et al*., 2011). Ethanolic extracts from *H. perforatum* were reported to exert inhibition effect on AChE (Altun *et al*., 2013). Some authors suggested this plant species to have a possible action on AChE in terms of the reduction of the degradation rate of ACh (Re *et al*., 2003). Despite, lower activity for methanolic extract has been reported (IC_{50} = 178 µg/mL) (Wszelaki *et al*., 2010). The neuroprotective activity of amentoflavone (biflavonoid), also found in *H. perforatum*, was first described in hypoxia/ischemia-induced neuronal injury both *in vitro* and *in vivo* systems. This compound can protect against Aβ1-42-induced neurological dysfunction in a rat model, which underscores its potential use in the prevention and treatment of AD and highlighting it as a clinical compound for the prevention and treatment of AD (Zhao *et al*., 2019). Other authors also reported that extracts of *H. perforatum* were able to improve cognitive function in $AlCl_3$-induced AD rats, by attenuating $AlCl_3$-induced increase in acetylcholinesterase activity and glutamic acid level, but also attenuating the increase of Aβ42 level, and also through the antioxidant and antiinflammatory activities (Zerrouki *et al*., 2016; Cao *et al*., 2017).

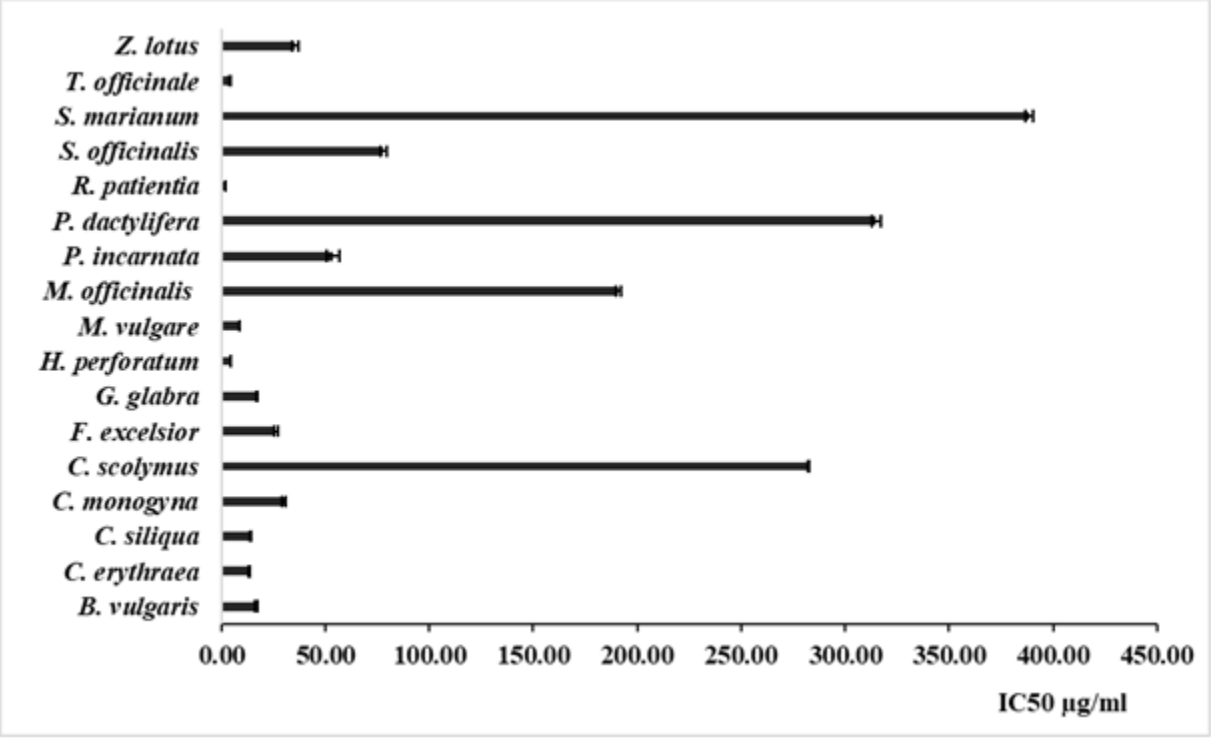

Figure No. 5
Acetylcholinesterase (AChE) inhibition of the studied species, results are expressed as (IC_{50} in mg/mL)

AChE inhibition activities from *M. vulgare* extracts which are mainly rich in flavonoids and phenylethanoids has been already demonstrated (Amessis-Ouchemoukh *et al*., 2014), although other authors have reported low inhibition of ethanolic extracts but high antioxidant activity due to the relative high amount of phenols (Salaj *et al*., 2018). Furthermore, *C. erythraea* extracts have been reported to be good AChE inhibitors and can block the acetylcholinesterase-induced β-amyloid

aggregation due to the presence of xanthones molecules (Guedes *et al*., 2019).

According to some authors, the ability of *B. vulgaris* extracts for inhibiting the AChE activity or with potential for the treatment of Alzheimer's disease is related with the presence of alkaloids and not phenols (Bonesi *et al*., 2013; Hostalkova *et al*., 2019), although other ones did not find this correlation (Kolar *et al*., 2010). In what concerns *G. glabra*, there are authors (Chakravarthi *et al*., 2013) who consider the root of this species as a promising drug for improving memory in the management of impaired learning, dementia, Alzheimer's disease, but there are other ones (Cui *et al*., 2008) who verified in *in vivo* studies that glabridin extracted from the roots of *G. glabra* able to reduce the cholinesterases activity in mice brain.

Correlation

A very strong linear correlation between ORAC antioxidant activity and both TPC (R^2 = 0.94) and TFC (R^2 = 0.90) was observed in the present study (Figure No. 6), suggesting that the most of the antioxidant activity of the plant extracts under study is due to the contribution of the phenolic and flavonoid compounds. Furthermore, the ORAC of the studied extracts was highly correlated with their inhibition of lipid peroxidation and reasonably correlated their AChE inhibition, indicating that those antioxidant compounds can probably act concurrently as lipid oxidation and AChE inhibitors. Moreover, a high positive correlation was found between LPI and TPC and a moderate positive correlation between LPI and TFC. Additionally, the high positive correlation was found between AChEi and LPI indicates that presumably the same compounds are responsible for the two activities.

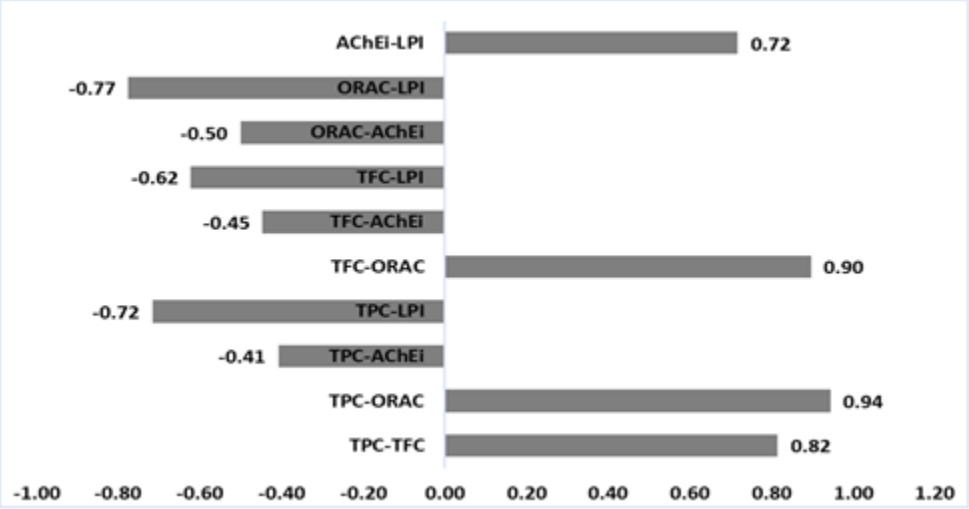

Figure No. 6
Correlations between the studied parameters

Principal component analysis (PCA) Analysis

Principal component analysis (PCA) was performed with a view to compare and classify the investigated medicinal plants according to their total phenolic and flavonoids contents (TPC and TFC), antioxidant activities (ORAC and LPI) in addition to their capacity to inhibit AChE. The results are shown in Figure No. 7. The PCA reduced the variables into two main principal components with components 1 and 2 explaining 75.523 and 15.926% variability, respectively. The two first components were able to explain 91.45% of variability among the plant extracts. PC1 was positively associated with all the five studied variables. The PCA score plot showed the projection and clear separation of five different groups. The leading or primary group contained *R. patiencia, H. perforatum,* and *T. officinale,* with the highest antioxidant activity strongly correlated with high TPC, TFC and ORAC antioxidant activity, along with their high efficiency to inhibit AChE and liposomes peroxidation, indicating their superiority compared the rest of the studied species. The second

group consisted of *B. vulgaris, C. erythraea, C. monogyna, G. glabra* and *S. officinalis.* Regarding the antioxidant activity, all species of this group, comes in the second order, which is characterized by good ORAC antioxidant activity ranging from 300 to 500 µMTE/g dw. This group is also distinguished good capacity to inhibit lipid peroxidation and AChE activity. The third PCA group consisted of *C. siliqua, F. excelsior, M. vulgare, Z. lotus* and *P. incarnata*. This group is characterized by low TPC and TFC along with low antioxidants activities. The forth PCA group consisted of only one plant *M. officinalis*, this species even ranked among the species with the lowest AChE inhibition it contains a moderate level of TPC and exhibited a good antioxidant activity, amount of TPC along with good AChE inhibitory activity. The fifth PCA group comprises *C. scolymus, P. dactylifera* and *S. marianum* with the lowest associated variables values. This group stand on the opposite side of the first group and was negatively correlated to all studied variables.

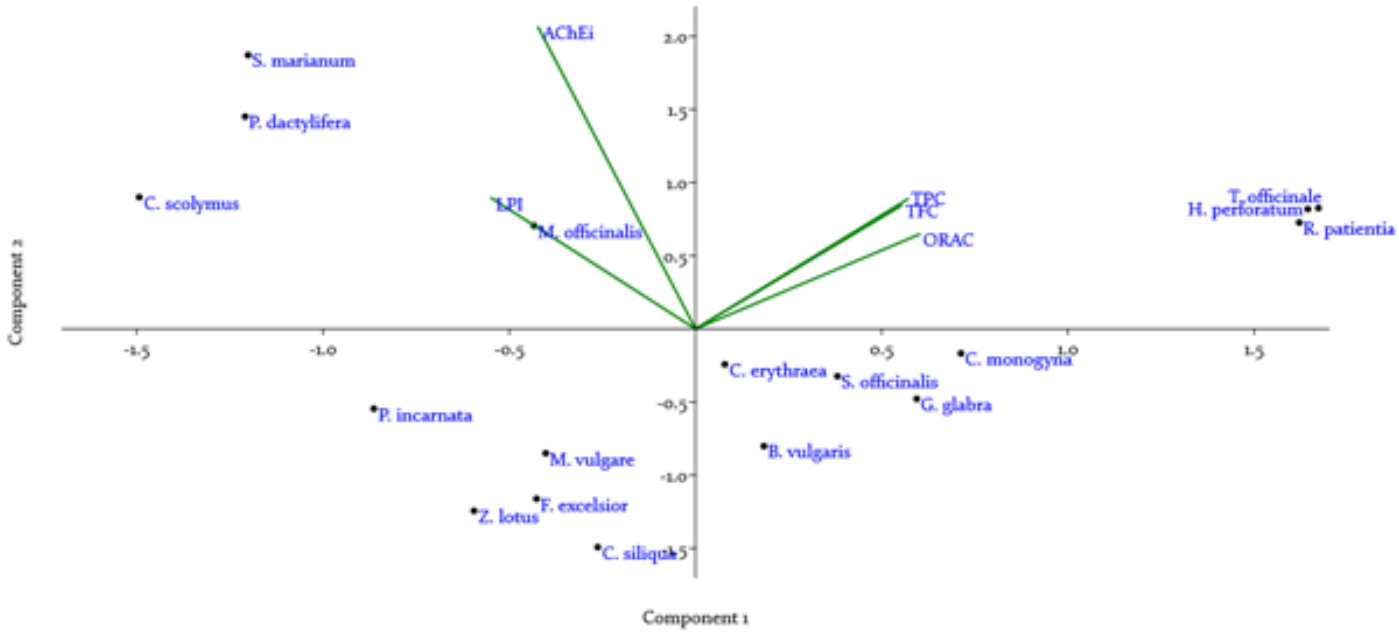

Figure No. 7
Principal component analysis (PCA) using the phenolic and flavonoid content and the in vitro biological properties of the six Moroccan plants. (a) Plot using the first and the second component

CONCLUSIONS

The results revealed that among the medicinal plants tested, extracts from R. *acetosa, H. perforatum* and *T. officinale* were the most effective for inhibiting AChE, besides presenting the highest total phenolic and flavonoids contents and being the best antioxidants and lipid peroxidation inhibitors along with *S. officinalis*. Hence, the highest efficiency in inhibition AChE along with strong antioxidant activity and lipid peroxidation inhibition demonstrated by these species reveal their potential as promising species for AD treatment. These three species were also identified as the most promising species using NJ cluster analysis and PCA analysis based on the studied parameters. Additionally, *M. vulgare, C. erythraea, C. siliqua, B. vulgaris* and *G. glabra* were very effective in inhibiting AChE, pointed out that *M. vulgare* was less efficient for LPI and having low TPC and TFC suggesting that its activity may be due to other phytochemicals than phenolic compounds.

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