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Enzyme Inhibitory and Antioxidant Activities of Different Extracts from *Ruscus aculeatus* L.

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ABSTRACT

The goal of this work was to evaluate *in vitro* antioxidant, anti-urease and anticholinesterase activities of *Ruscus aculeatus* L. using a variety of extracts. Antioxidant activities of different extracts from plant were evaluated using DPPH, FRAP, ABTS/TEAC and CUPRAC methods. In addition, the anti-urease and anticholinesterase activities of the different extracts were compared by Indophenol and Ellman tests, respectively. In the present study, Soxhlet chloroform extract showed stronger antioxidant (DPPH, FRAP, CUPRAC) and anticholinesterase activity than other extracts. It was also found that the maceration ethanol extract showed the most potent anti-urease activity. The Soxhlet chloroform and maceration ethanol extracts from this species may be a natural resource candidate for the pharmaceutical and food industry due to the pharmacological effects (antioxidant, anticholinesterase and anti-urease effect).

Key words: *R. aculeatus;* antioxidant, anti-urease, anticholinesterase

INTRODUCTION

In the biological system, reactive oxygen species (ROS) and nitrogen types (RNS) can damage DNA and lead to oxidation of lipids and proteins in cells. Normally, the antioxidant system occurring in the human body can clear these radicals, but exposure to smoking, alcohol, radiation or environmental toxins induces excess ROS and RNS production. Increased intake of exogenous antioxidants reduces the effects caused by these radicals. Natural antioxidants are

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commonly found in food and medicinal plants. These natural antioxidants have a wide range of biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis and anticancer [1-4].

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases. It is related with memory, learning abilities and life quality of individuals. This disease is expressed by the presence of β-amyloid plaque, intracellular neurofibrillary tangles (NFTs), synaptic deterioration, and neuronal death[5,6]. To treat of AD is is used rivastigmine, galantamine, donepezil, memantine, memantine combined with donepezil, tacrine and memantine but none of the pharmacologic treatments available today for Alzheimer's dementia slow or stop the damage and destruction of neurons that cause Alzheimer's symptoms and make the disease fatal [7].

Helicobacter pylori (HP) usually colonizes in the surface of human gastric mucosa and duodenal bulb. After that releases urease that converts urea into ammonia. This enzyme produces an alkaline environment that makes it suitable for bacterial growth and the manifestation of the disease. *H.pylori* causes chronic gastritis, gastric carcinoma, duodenal ulcer in more than 50% of people. At the same time in more than %80 of people with *H.pylori* are asymptomatic. Therefore it is the most critical widespread infection in the world and plays a big role in maintaining stomach ecology [8]. It has been tested that a urease-negative mutant does not cause gastritis due to difficulties in colonization, therefore, specific inhibition of urease activity has been proposed as a successful strategy to eliminate the organism in the body. To treat of *H. pylori* it is used triple therapy, which includes a proton pump inhibitor and any of the two antibiotics such as amoxicillin (AMX), clarithromycin (CLA), metronidazole (MNZ) and tetracycline (TET) [9].

Medicinal herbs have historically been a valuable source of therapeutic agents, and most of the drugs used today are natural products of plant origin or derivatives thereof. According to the World Health Organization (WHO), over 80% of the world population uses traditional medicine for primary health care. The number of studies on these has increased in recent years as natural products such as plant extracts, either as pure compounds or as standardized extracts offer unlimited opportunities for new drug discoveries [10-13].

Ruscus aculeatus L. (butcher's broom) is belong to the Asparagaceae family and it grows wildly in the forests in Mediterranean Europe and Africa. It has tough, green, erect, striated stems and very rigid leaves. The small greenish-white flowers grow from the center of the leaves and bloom in the early spring. The thick root, typically collected in autumn, is used medicinally. *R. aculeatus* includes

steroidal saponins, ruscogenin, neoroscogenin, essential oils, flavonoids, resin and minerals. This plant is used for treatment of venous insufficiency / varicosities edema such as circulatory disorders, edema, thrombophlebits, swelling and also used as diuretic. Also it is used in premenstrual syndrome, hemorrhoids, diabetic retinopathy, skin disease, against inflammation and arthritis [14-16].

The best of our knowledge, there are no reports on the effect of extraction methods on the biological activity (antioxidant, anti- urease, and anticholinesterase) of this plant. For this reason, the aim of this work was to evaluate *in vitro* antioxidant, anti-urease and anticholinesterase activities of *R. aculeatus* using a variety of extracts.

METHODOLOGY

Identification of plant material

Ruscus aculeatus L. was taxonomically identified by Dr. Gizem Bulut. The voucher specimens, representative samples of the plant material, were archived in the herbarium of the Faculty of Pharmacy, Marmara University and documented with the herbarium number of MARE:19140.

Preparation of *Ruscus aculeatus* extracts

Aerial parts of *Ruscus aculeatus* were dried at 25°C in the shade. Dried parts of the plant were treated with a mechanical grinder (Renas, RBT1250) for fine powder and proper weight. The two extraction methods were performed to gain crude extracts from the aerial parts of the plant. **Maceration:** Plant powder (20 g) was extracted with the use of petroleum ether, chloroform, and ethanol until colorless. **Soxhlet extraction:** 20 gram of plant powder was extracted in Soxhlet apparatus with petroleum ether, chloroform and ethanol. The six different extracts from plant were concentrated by rotary vacuum evaporator. All the extracts obtained were stored at 4°C for future analysis.

In vitro bioactivity assays

2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay

240 µL 0.1 mM DPPH solution was added to 10 µL sample of the extracts at different concentrations (0.5-5 mg/mL). Prepared mixture was stirred for 1 min. and placed at 25°C for 30 min. The mixture absorbance was determined against the reference at 517 nm. Control sample was carried out under the same conditions using 10 µL of methanol instead of experimental and standard materials and the control sample was daily measured. The investigation was performed three times and the averages of the data and standard deviation were calculated. The data gained from the investigation was given as IC_{50} =mg/mL [17].

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\cdot+}$) scavenging assay

50 µL of extracts prepared at different concentrations (1–5 mg/mL), 50 µL of $ABTS^{\cdot+}$ working solution and 150 µL distilled water were added on the prepared extracts. The mixture absorbance was determined against the reference at 734 nm for 6 min. Control sample was prepared under the same conditions with the use of 50 µL distilled water instead of experimental and standard materials. The control sample was daily measured. ABTS radical scavengimg determination was applied to trolox solutions prepared at different concentration (0.2-1 mM). The results from this study were given as mM trolox/g extract [18].

Ferric reducing/antioxidant power (FRAP) assay

The method of Benzie and Strain (1996) was applied to the extracts in order to estimate the ferric reducing ability. The FRAP reagent [25 mL 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution and 2.5 mL 20 mM $FeCl_3 \cdot 6H_2O$] was kept at 37°C for 30 min. 190 µL FRAP reagent was mixed with 10 µL extract and the mixture absorbance was determined at 593 nm after 4 min. FRAP values of the extracts were given as mM Fe^{2+}/mg extract [19].

Cupric ion reducing/antioxidant power (CUPRAC) assay

60 µL Cu(II)x2H_2O, 60 µL neocuproine and 60 µL, NH_4Ac (1 M) were mixed. Then 60 µL of the extract and 10 µL of ethanol were added to the mixture. After the duration time of 60 min, the mixture absorbance was spectrophotometrically measured at 450 nm. CUPRAC values of the extracts were given as mM trolox/mg extract [20].

Anti-urease activity assay

Stock solutions were prepared from different extracts obtained from the plant and these solutions were diluted to prepare working solutions. Working solution (100 µL) was taken and urease (500 µL) was added on it. The mixture was incubated at 37°C for 30 min. Then, 1100 µL of urea was added on this mixture and kept in the incubator at 37°C for 30 min. R1 (1% phenol, 0.005% sodium nitroprusside) and R2 (0.5% NaOH, 0.1% sodium hypochlorite) reagents were added to the mixture, respectively. After the incubation period at 37°C for 2 h, the absorbance of samples was measured at 635 nm [21].

The % inhibition of urease was calculated by the formula:

% enzyme inhibition = $[(A_o - A_i)/A_o] \times 100]$

A_o: The absorbance of the control solution

A_i: Absorbance of plant extracts and standard solutions.

Anticholinesterase activity assay

Inhibition activities of acetylcholinesterase (AchE) were measured using microplate reader. Acetylcholinesterase as enzyme source derived from electric fish, acetyl thiokolin iodide was used as substrate. Yellow-colored 5,5-dithiobis- (2-nitrobenzoic acid) (DTNB) was used for the measurement of activity. As a control, ethanol and galantamine, the alkaloid type drug isolated from the galanthus plant, were used as controls.

AChE % Inhibition Test

AChE (20 μL) and different concentrations of extracts (20 μL) were added to phosphate buffer solution (pH 8.2 0.1 M, 40 μL). This mixture was incubated at 25^{0}C for 10 min. After incubation, DTNB (100 μL) and AcI (20 μL) as substrate were added on the mixture. The same procedure was applied to the galantamine used as standard. 5-thio-2-nitrobenzoic acid was spectrophotometrically measured at 412 nm. Anticholinesterase activity of the extracts was calculated using the following equation as% inhibition relative to control [22].

%I = (Acontrol – Asample/Acontrol)x100

Statistical analysis

All the experiments were done in triplicates. The results of the antioxidant, anticholinesterase and anti-urease experiments were demonstrated as mean ± SD. All the data was analysed by the Graphpad Prism 5 program. Statistical differences between the study groups were analysed using two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test and p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In vitro evaluation of biological activity

Antioxidant activity of extracts

The antioxidant activities of plant's different extracts were shown in Table 1. The Soxhlet chloroform (IC_{50} 0.18 mg/mL) extract showed the strongest DPPH free radical scavenging activity. The petroleum ether extracts obtained from Soxhlet and maceration extraction methods showed the lowest DPPH free radical scavenging activity. As shown in Table 1, the radical scavenging DPPH activities of all extracts showed lower than that of ascorbic acid (IC_{50}: 0.005 mg/mL) and BHA (IC_{50}: 0.006 mg/mL). When the results of all extracts were compared for ABTS radical cation scavenging activity, it found that ethanol extracts obtained from Soxhlet (3.24 mM trolox/g extract) and maceration (3.22

mM trolox/g extract) extraction methods exhibited the strongest ABTS radical cation scavenging activity. Soxhlet chloroform (0.37 mM Fe^{2+}/mg extract) and maceration chloroform extracts (0.33 mM Fe^{2+}/mg extract) showed stronger ferric reducing activity than other extracts. When the results of all extracts and standard were compared, it found that all extracts exhibited lower ferric reducing activity than BHT.

The chloroform extracts obtained from Soxhlet (0.86 mM trolox/mg extract) and maceration (0.50 mM trolox/mg extract) extraction methods exhibited stronger cupric reducing antioxidant activity than other extracts. When the results of the CUPRAC assay were examined, the all extracts showed lower cupric reducing antioxidant activity than BHA compounds.

The results obtained from this study showed that Soxhlet extraction techniques are the most suitable method to get the most powerful DPPH, ABTS, FRAP and CUPRAC antioxidant activity. It was also found that the most suitable solvent for obtaining high DPPH, FRAP and CUPRAC values was chloroform.

Table 1. Effects of extracting solvents/methods on the antioxidant activity of *Ruscus aculeatus* extracts

	DPPH (IC_{50}: mg/mL)		ABTS (mM trolox/g extract)		FRAP assay (mM Fe^{2+}/mg extract)		CUPRAC assay (mM trolox/mg extract)	
Samples	Soxhlet	Maceration	Soxhlet	Maceration	Soxhlet	Maceration	Soxhlet	Maceration
Petroleum ether	2.32±0.08[a]	0.83±0.02[a]	1.63±0.01 a	1.56±0.03[a]	0.15±0.01[a]	0.10±0.001[a]	0.35±0.01[a]	0.25±0.002[a]
Chloroform	**0.18±0.01[b]**	0.81±0.02[b]	1.92±0.13 [b]	3.10±0.02[b]	**0.37±0.02[b]**	0.33±0.002[b]	**0.86±0.002[b]**	**0.50±0.005[b]**
Ethanol	0.26±0.04[c]	0.79±0.10[c]	**3.24±0.01 [c]**	**3.22±0.01[c,b]**	0.12±0.03[c,a]	0.32±0.004[c,b]	0.11±0.01[c]	0.15±0.004[c]
Ascorbic acid	0.005±0.009[d] 0.005±0.009[d]		13.01±0.01[d] 13.01±0.01[d]					
BHT					1.1±0.12[d]	1.1±0		
BHA	0.006±0.006[e]	0.006±0.					1.62±0.12[d] 1.62±0.12[d]	

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts [(a-d)] are significantly different, $p<0.05$.

Urease inhibitory activity

The percentage of inhibition of urease enzyme at 12.5 µg/mL concentration of different extracts was determined by using indophenol method and the results were shown in Table 2. In Soxhlet method, ethanol (29.19 %) extract showed stronger anti-urease activity than other extracts. In maceration method, ethanol (34.24 %) extract exhibited the highest anti-urease activity. The petroleum ether obtained from both extraction methods did not show anti-urease activity in this study. Comparing the activity results of all the extracts, it was found that maceration ethanol extract had the strongest anti-urease activity and all extract showed lower activity than thiourea compounds (78.84%). The results showed that maceration extraction technique is the most suitable method to obtain the strongest anti-urease activity. It was also found that the most suitable solvent for obtaining strong anti-urease activity was ethanol.

Table 2. The urease inhibitory activity of different extracts from *Ruscus aculeatus*

Samples	Urease inhibition (%) (12.5 µg/mL)	
	Soxhlet	Maceration
Petroleum ether	NA	NA
Chloroform	19.09±1.2[a]	19.09±1.2[a]
Ethanol	29.19±1.8[b]	29.19±1.8[b]
Galantamine	78.84±0.9[c]	78.84±0.9[c]

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts [(a-c)] are significantly different, $p<0.05$; NA: not activity

Anticholinesterase activity

The percentage of inhibition of cholinesterase enzyme at 500 µg/mL concentration of different extracts was determined by using Ellman method and the results were shown in Table 3. In Soxhlet method, chloroform (94.37%) extract exhibited higher percentage of inhibition of cholinesterase enzyme than other extracts. In maceration method, ethanol (80.87%) extract showed the strongest anticholinesterase activity. The Soxhlet petroleum ether and maceration petroleum ether extracts did not show cholinesterase inhibitory activity. In the present study, Soxhlet chloroform method/solvent were the most suitable solvent and method to get the strongest anticholinesterase activity. The compounds or extracts having strong antioxidant activity is generally known to have strong anticholinesterase activity. In this study, Soxhlet chloroform extract showing strong antioxidant activity (DPPH, FRAP, CUPRAC) in parallel with the literature also showed strong anticholinesterase activity.

Table 3. The anticholinesterase activity of different extracts from *Ruscus aculeatus*

Samples	Enzyme inhibition (%) (500 µg/mL)	Enzyme inhibition (%) (500 µg/mL)
	Soxhlet	Soxhlet
Petroleum ether	NA	NA
Chloroform	94.37±1.2[a]	94.37±1.2[a]
Ethanol	84.35±1.3[b]	84.35±1.3[b]
Galantamine	96.54±0.9[c,a]	96.54±0.9[c,a]

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts [(a-c)] are significantly different, $p<0.05$; NA: not activity.

Literature information on the antioxidant activity of aerial parts of *R. aculeatus* are scarce, while there not any anti-urease and anticholinesterase activity for this species. In a study, methanol, ethyl acetate and butanol extracts from the aerial parts of the plant and their antioxidant and antimicrobial activities were examined. According to the obtained data, the fraction ethyl acetate (IC_{50}:158 µg/mL) and butanol (IC_{50}:173 µg/mL) extracts were found to have the strongest DPPH radical scavenging activity. In addition, it was found that fraction ethyl acetate extract showed stronger antimicrobial activity than other extracts[14]. When we compared the results above with our results, it was found that the Soxhlet chloroform (IC_{50}:180 µg/mL) extract was close to the butanol extract (IC_{50}:173 µg/mL), but all extracts obtained from both extraction methods exhibited lower DPPH radical scavenging activity than ethyl acetate and butanol extracts.

In another study, ethanol, acetone and ethyl acetate extracts obtained using Soxhlet method from the aerial parts of the plant and antioxidant activities of these extracts were investigated. In this study, acetone and ethyl acetate extracts showed the best total antioxidant capacity (23.329 µg AA g^{-1}) and the highest DPPH scavenging activity (IC_{50} = 182.54 μg mL^{-1}) respectively[16].

When we compared the results of this study with the results of the literature, it was found that the Soxhlet chloroform (IC_{50}:180 µg/mL) extract showed DPPH radical scavenging activity very close to the ethyl acetate (IC_{50} = 182.54 μg mL^{-1}) extract.

Luis et al. investigated the antioxidant activity of methanol extract obtained using Soxhlet method from the plant's aerial parts and found that this extract showed strong DPPH (IC_{50} 0.172 mg/mL) radical scavenging activity [23]. According to the data obtained in this study, Soxhlet ethanol extract (IC_{50} 0.26 mg/mL) obtained in our study showed lower DPPH radical scavenging activity than the methanol extract (IC_{50} 0.172 mg/mL) in the literature. Moreover, no stud-

ies on the anti-urease and anticholinesterase activity of the plant were found in the literature review and the anti-urease and anticholinesterase activities of the different extracts from the plant were examined for the first time in this study.

Consequently, according to the results of this study, Soxhlet chloroform extract showed stronger antioxidant (DPPH, FRAP, CUPRAC) and anticholinesterase activity than other extracts. It was also found that the maceration ethanol extract showed the most potent anti-urease activity. Therefore, Soxhlet chloroform and maceration ethanol extracts from this species may be a natural resource candidate for the pharmaceutical and food industry due to the pharmacological effects (antioxidant, anticholinesterase and anti-urease effect).

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided here in.

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REFERENCES

1. Xu, D.P.; Li, Y.; Meng, X.; Zhou, T.; Zhou, Y.; Zheng, J.; Zhang, J.J.; Li1, H.B. Natural antioxidants in foods and medicinal plants: extraction, assessment and resources. *Int J Mol Sci* **2017**. *18(1)*, 96-99.

2. Peng, C.; Wang, X.; Chen, J.; Jiao, R.; Wang, L.; Li, Y.M.; Zuo, Y.; Liu, Y.; Lei, L.; Ma, K.Y.; Huang, Y.; Chen, Z.Y. Biology of ageing and role of dietary antioxidants. *BioMed Res Int* **2014**. *2014*, 1-13.

3. Li, S.; Tan, H.Y.; Wang, N.; Zhang, Z.J.; Lao, L.; Wong, C.W.; Feng, Y. The role of oxidative stress and antioxidants in liver diseases. *Int J Mol Sci* **2015**. *16(11),* 26087-26124.

4. Wang, F.; Li, Y.; Zhang, Y.J.; Zhou, Y.; Li, S.; Li, H.B. Natural products for the prevention and treatment of hangover and alcohol use disorder. *Molecules***. 2016**. *21(1),* 1-21.

5. Bostancıklıoğlu, M. An update on the interactions between Alzheimer's disease, autophagy and inflammation. *Gene* **2019**. *705*, 157-166.

6. Ulep, M.G.; Saraon, S.K.; McLea, S. Alzheimer Disease. *J Nurse Pract* **2018**. *14*, 129-135.

7. Colović, M.B.; Krstić, D.Z; Lazarević-Pašti, T.D.; Bondžić, A.M.; Vasić, V.M. Acetylcholinesterase inhibitors: pharmacology and toxicology. *Curr Neuropharmacol* **2013.** *11*, 315-335.

8. Yao, Y.; Shen, Y.; Zhu, L.; Ni, Y.; Wang, H.; Shao, S. Preliminary study and bioinformatics analysis on the potential role of CagQ in type IV secretion system of *H.pylori*. *Microb Pathog* **2018**. *116*, 1-7.

9. Amin, M.; Anwar, F.; Naz, F.; Mehmood, T.; Saari, N. Anti-*Helicobacter pylori* and urease ınhibition activities of some traditional medicinal plants. *Molecules* **2013**. *18*, 2-8

10. Atanasov, A.G.; Waltenberger, B.; Pferschy-Wenzig, E.M.; Linder, T.; Wawrosch, C.; Uhrin, P.; Temm, V.; Wang, L.; Schwaiger, S.; et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol Adv* **2015**. *33(8),*1582-1614.

11. Kinghorn, A.D.; Pan, L.; Fletcher, J.N.; Chai, H. The relevance of higher plants in lead compound discovery programs. *J Nat Prod* **2011**.*74*,1539-1555.

12. Sasidharan, S.; Chen, Y.; Saravanan, D.; Sundram, K.M.; Latha, L.Y. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afr J Tradit Complement Altern Med.* **2011**. *8(1),* 1-10.

13. Duraipandiyan, V.; Ayyanar, M.; Ignacimuthu, S. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC Complementary Altern Med.* **2006.** *6*, 35-41.

14. Hadžifejzović, N.; Kukić-Marković, J; Petrović, S.; Soković, M.; Glamočlija, J.; Stojković, D.; Nahrstedt, A. Bioactivity of the extracts and compounds of *Ruscus aculeatus* L. and *Ruscus hypoglossum* L. *Ind Crop Prod* **2013**. *49*, 407-411.

15. Maswadeh, H.M.; Semreen, M.H.; Naddaf, A.R. Anti-inflammatory activity of *Achillea* and *Ruscus* topical gel on carrageenan-induced paw edema in rats. *Acta Pol Pharm* **2006**. *63*, 277-80.

16. Jakovljevic, V.D.; Milicevic, J.M.; Djelic, G.T.; Vrvic, M.M. Antioxidant activity of *Ruscus* species from Serbia; Potential new sources of natural antioxidants. *Hem ind* **2016.** *70*, 99-106.

17. Wei, F.C.; Jinglou, C.; Yongfang, C.; Liming, P. L. Antioxidant, free radical scavenging, anti-inflammatoryand hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch.etSav.) Ching. *J Ethnopharmacol* **2010**. *130*, 521-528.

18. Re, R.N.; Pellegrını, A.; Proteggente, A.; Pannala, M.; Yang, C.R.Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio Med* **1999**. *26,* 1231-1237.

19. Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* **1996**. *239*, 70-76.

20. Apak, R.; Güclü, K.; Ozyurek, M.; Karademir, S.E. 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* **2004**. *52 (2004),* 7970-7981.

21. Ghous, T.; Akhtar, K.; Nasim, F.U.H.; Choudhry, M.A. Screening of selected medicinal plants for urease inhibitory activity. *Biol Med* **2010**. *2,* 64-69.

22. Ellman, G.L.; Courtney, K.D.; Andress, V.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **1961**. *7*, 88-95.

23.Luís, Â.; Fernanda, D.; Duarte, A.P. Bioactive compounds, rp-hplc analysis of phenolics, andantioxidant activity of some portuguese shrub species extracts. *Nat Prod Commun* **2011**. *6 (12*), 15-32.