

NEW INSIGHT INTO ENZYME INHIBITORY
AND ANTI-PROLIFERATIVE ACTIVITY
OF *ECHINOPS RITRO* L. (ASTERACEAE)

Vessela Balabanova[#], Dimitrina Zheleva-Dimitrova,
Yulian Voynikov*, Gokhan Zengin**, Olivier Joubert***,
Reneta Gevrenova

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Abstract

Echinops ritro L. is a perennial herbaceous plant in the tribe Cardueae, Asteraceae family, widely distributed, up to 1000 m a.s.l. In the present study, the enzyme inhibitory and anti-proliferative activity of *E. ritro* leaves and flowering heads extracts were assayed. Quantitative determination of 22 analytes, including eleven acylquinic and one hydroxycinnamic acids, and ten flavonoids was performed by Ultra high-performance liquid chromatography – diode array detector (UHPLC-DAD) analyses. The major compound was 1,5-dicaffeoylquinic acid, being presented at 20.48 ± 0.22 µg/mg in the flowering heads, while the leaves demonstrated very high levels of chlorogenic acid (117.29 ± 14.20 µg/mg) and 3,5-dicaffeoylquinic acid (12.79 ± 0.282 µg/mg). The flavonoids were dominated by isoquercitrin and rutin (3.13 ± 0.033 and 3.25 ± 0.057 µg/mg, respectively) together with methoxylated aglycones nepetin, hispidulin and jaceosidin (up to 0.51 µg/mg). The leaves extract revealed prominent acetylcholinesterase (2.33 ± 0.09 mg GALAE/g) and α-glucosidase inhibitory potential (0.37 ± 0.01 mmol ACAE/g). Nevertheless, the flowering heads extract showed distinct butyrylcholinesterase (1.41 ± 0.35 mg GALAE/g), tyrosinase (45.66 ± 0.84 mg KAE/g), amylase (0.37 ± 0.02 mmol ACAE/g), and lipase (9.06 ± 1.43 mg OE/g) inhibitory activity. The anti-proliferative assay

[#]Corresponding author.

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was completed towards THP-1 monocyte leukemic cells, where at a concentration $> 1000 \mu\text{g/mL}$, a significant dose dependent reduction of cell viability was observed for the leaves extract. In conclusion, the data revealed a new insight into the taxon for its health benefit application.

Key words: *Echinops ritro*, enzyme inhibitory potential, anti-proliferative activity

Introduction. The genus *Echinops* L. (Asteraceae) includes about 130 species with wide range distribution at different habitats, up to 1000 m a.s.l. [1–4]. The taxon belongs to tribe Cardueae and the species are characterized by the presence of uniflowered capitula aggregated into second-order spherical or oval heads [3]. Based on the literature survey on chemical profile of *Echinops* species, the thiophenes (5-(but-3-en-1-ynyl)-2,2'-bithiophene, α -terthiophene, etc.) are the main constituents for the roots, while the aerial parts are rich in terpenes (sesqui- and triterpenoids), flavonoids and other phenolic compounds as apigenin, luteolin, nivegin, 7-hydroxyisoflavone, kaempferol, etc. [4]. Recently, phenylethanoid glycosides, hydroxycinnamoyl hexoses (sugar esters), a series of hydroxybenzoic and hydroxycinnamic acid glycosides, coumaroyl- and feruloylquinic acids, caffeoyl-hydroxydihydrocaffeoylquinic acids, caffeoyl-feruloylquinic, caffeoyl-dehydrocaffeoylquinic acids, tricafeoylquinic acid, dicafeoylquinic acid hexosides have been reported for *E. ritro* L. for the first time. Additionally, flavonoid rutinosides and acetylhexosides, methoxylated flavonoids, and their glycosides are newly reported for the species [5].

In line with the established secondary metabolites, different activities have been established for *Echinops* species. Based on the phenolic compounds, a profound study on antioxidant and hepatoprotective potential of *E. ritro* leaves and flowering heads extracts on induced oxidative stress in vitro/in vivo was carried out [5]. Moreover, enzyme inhibitory activity of the last-mentioned species was examined towards tyrosinase, α -amylase, α -glucosidase, acetyl- and butyrylcholinesterase [6]. The presence of thiophenes determines insecticidal, anti-proliferative and anti-fungal effects while antioxidant, anti-inflammatory and hepatoprotective activities are based on flavonoids and phenolic content [4]. The effect on cancer cell line has been tested too. Some results revealed a cytotoxic effect of different *Echinops* species towards human monocytic leukemia cell line (THP-1), hepatocarcinoma (HepG2 and MFC-7), acute myeloid and chronic myelogenous leukemia cell lines (HL-60 and K562, respectively) [7–9]. The anti-proliferative activity against HT-29 cell lines and tumour cell lines as HepG2, HeLa, HT-29 and A549 was carried out too [10,11].

Notwithstanding studies on the anti-proliferative activity effect of any *Echinops* species, *E. ritro* L. has not been thoroughly investigated on this activity. Moreover, an additional detailed investigation on the main phenolic acids and flavonoids, and the enzyme inhibitory potential is not carried out and it seems essential.

In the present study, the enzyme inhibitory capacity and cytotoxicity of *E. ritro* leaves and flowering heads extracts together with quantitative determination of the main phenolic compounds were done. Thus, the investigation will bring a new insight into the knowledge of this species.

Materials and methods. Plant material. *Echinops ritro* plant material was collected at Kardzhali region, Eastern Rhodopes, Bulgaria (41.6447° N, 25.3749° E), during full flowering stage, August 2020. The plant was identified by one of us (D. Zh.) according to STOJANOV [3]. A voucher specimen was deposited at the Herbarium Academiae Scientiarum Bulgariae (SOM 178490). The plant samples were separated into leaves and flowering heads, and afterwards dried at room temperature.

Sample extraction. Air-dried powdered leaves (100 g) and flowering heads (100 g) were extracted twice with 80% MeOH (1:20 w/v) by sonication (80 kHz, ultra-sound bath Biobase UC-20C) for 15 min at room temperature. Then, the extracts were concentrated in vacuo and subsequently lyophilized (lyophilizer Biobase BK-FD10P) to yield crude extracts of 5.10 g and 4.51 g for the leaves and flowering heads, respectively.

Chemicals and reagents. Neochloreogenic, chlorogenic acid (**2**), *p*-coumaric acid (**3**), isoquercitrin (**4**), rutin (**5**), hyperoside (**6**), isorhamnetin 3-*O*-rutinoside (**7**), 3,4-diCQA (**9**), 1,5-diCQA (**10**), 3,5-diCQA (**11**), luteolin (**14**), nepetin (**15**), apigenin (**16**), hispidulin (**18**), jaceosidin (**19**) were provided from Extrasynthese (Genay, France).

UHPLC-DAD analysis. The UHPLC-DAD analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 analytical system equipped with a Dionex UltiMate 3000 RS Pump (LPG-3400RS), Dionex UltiMate 3000 RS Autosampler (WPS-3000TRS), Dionex UltiMate 3000 RS Column Compartment (TCC3000RS) and Dionex UltiMate 3000 Diode Array Detector (DAD-3000). The separation was achieved on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters), with mobile phase consisting of A water (with 0.1% formic acid) and B acetonitrile (0.1% formic acid). The used gradient was as follows: 0 min 5% B, 1 min 5% B, 20 min 30% B, 25 min 70% B, 33 min 95% B. The system was then turned to the initial condition and equilibrated over 6 min. The flow rate was 300 μL/min and the injection volume was 1 μL. The UV wavelengths were 310 nm (phenolic acids) and 360 nm (flavonoids). The entire system was controlled by Chromeleon software, version 7.2.

Quantitative analysis. The high-performance liquid chromatography (HPLC) analysis of phenolic acids (**1**, **2**, **3**, **8**, **9**, **10**, **11**, **12**, **13**, **17**, **20** and **21**) and flavonoids (**4**, **5**, **6**, **7**, **14**, **15**, **16**, **18**, **19** and **22**) was carried out using the external standard method. Standard calibrations of **2**, **3**, **5**, **7**, **9**, **10** and **16** were established at five data points covering the concentration range of each analyte according to the level expected in the plant samples. Working solutions containing 0.2, 0.1, 0.05, 0.02 and 0.01 mg/mL of the assayed phenolic

acids were prepared from a stock solution in methanol containing 0.5 mg/mL. Triplicate analyses were performed for each concentration and the peak area was detected at 310 nm (for phenolic acids) and 360 nm (for flavonoids). Based on the similar UV spectra, the quantity of neochlorogenic acid (**1**) was determined based to the calibration curve of chlorogenic acid (**2**), isoquercitrin (**4**) and hyperoside (**6**) as rutin (**5**), luteolin (**14**), nepetin (**15**), hispidulin (**18**), jaceosidin (**19**), and cirsilineol (**22**) as apigenin (**16**), while diacyquinic acids (diAQA) (**11**, **12**, **13** and **17**) as 3,4-dicaffeoylquinic acid (diCQA) (**9**).

Determination of enzyme inhibitory effects. Extracts (0.2–1 mg/ml) were assayed for evaluating enzyme inhibition effects towards acetyl- and butyrylcholinesterase inhibitory activity, as well as tyrosinase, α -amylase, α -glucosidase and lipase. Detailed procedures were reported elsewhere [12,13].

Cell line and culture. The human monocytic THP-1 (TIB-202) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells grown in RPMI 1640 were supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin and 0.5% amphotericin B. Cells were cultured in a humidified atmosphere at 37 °C, under a 5% CO₂ atmosphere [14].

Cytotoxicity assay. THP-1 cells (at 1.105 cells/ml) cells in RPMI medium (Thermo-Fisher) supplemented with 10% FBS were seeded in each well of a 48-well plate ($n = 4$). Cells were permitted to adhere for 24 h, and then treated with leaves and flower heads *E. ritro* extracts in a medium for 24 h. Subsequently, 40 μ L of WST-1 testing solutions (Sigma-Aldrich) was added to each well and the plate was incubated at 37 °C for 2 h. The contents of each well were laid down in three wells of a 96-well plate. The absorbances were measured at 450 and 630 nm with an Omega StarLab spectrophotometer (Omega, Ortenberg, Germany).

Results and discussion. UHPLC-DAD analysis. An HPLC-DAD method was developed for the quantitative determination of the main phenolic acids and flavonoids in *E. ritro* extracts. Based on the common approach, HPLC analysis of the analytes was performed with mobile phase composed of acidified with formic acid acetonitrile and water [5]. Analyte's identification was performed by comparison of retention times and UV-spectra with reference standards. A total of 22 analytes, including eleven acylquinic acids (**1**, **2**, **8**, **9**, **10**, **11**, **12**, **13**, **17**, **20** and **21**), one hydroxycinnamic acid (**3**), and ten flavonoids (**4**, **5**, **6**, **7**, **14**, **15**, **16**, **18**, **19** and **22**) were determined in *E. ritro*. Typical HPLC-DAD chromatograms of *E. ritro* leaves and flowering heads extracts are presented in Fig. 1. Analytical characteristics of the HPLC-UV method are presented in Table 1. The linear regression for the calibration curves showed good linear relationships with $r^2 > 0.992$ within the test ranges. The content of the assayed compounds is revealed in Table 2.

The main compound in the tested flowering heads extract was 1,5-diCQA (**10**), followed by chlorogenic acid (**2**), while the leaves demonstrated very high level of chlorogenic acid (**2**) and 3,5-diCQA (**11**). Moreover, leaves extract re-

T a b l e 1

Regression equation, limit of detection (LOD), limit of quantification (LOQ),
obtained for the compounds

No.	Analyte	t _R	UV	Equation	R ²	LOD (µg/ml)	LOQ (µg/ml)
2	Chlorogenic acid	7.39	310	$y = 0.034x + 6.8296$	0.9811	0.97	2.94
3	<i>p</i> -coumaric acid	11.10	310	$y = 3.301x - 14.713$	0.9994	0.13	0.40
5	Rutin	14.05	360	$y = 0.0503x + 0.6374$	0.9992	0.66	1.99
7	Isorhamnetin 3- <i>O</i> -rutinoside	15.84	360	$y = 9.4672x - 1.3959$	0.9998	0.76 ng/ml	2.32 ng/ml
9	3,4-diCQA	15.87	310	$y = 0.0939x + 0.1103$	0.9996	0.19	0.57
10	1,5-diCQA	16.02	310	$y = 0.1117x - 0.0322$	0.9995	0.17	0.50
16	Apigenin	23.01	360	$y = 1.3856x + 0.7466$	0.9907	3.1 ng/ml	9.2 ng/ml

T a b l e 2

Content (in µg/mg dry extract) of compounds assayed in the studied *E. ritro* extracts

No.	Analyte	t _R	Flowering heads (µg/mg de)	Leaves (µg/mg de)
1	Neochlorogenic acid	3.93	under LOQ	under LOQ
2	Chlorogenic acid	7.41	13.37 ± 4.82	117.29 ± 14.20
3	<i>p</i> -coumaric acid	11.10	0.52 ± 0.002	—
4	Isoquercitrin	14.02	—	3.13 ± 0.033
5	Rutin	14.43	—	3.25 ± 0.057
6	Hyperoside	14.70	—	0.23 ± 0.004
7	Isorhamnetin 3- <i>O</i> -rutinoside	15.55	—	0.027 ± 0.0005
8	diAQA1	15.69	0.56 ± 0.060	—
9	3,4-diCQA	15.85	0.80 ± 0.004	0.97 ± 0.009
10	1,5-diCQA	15.99	20.48 ± 0.22	0.88 ± 0.009
11	3,5-diCQA	16.11	8.10 ± 0.10	12.79 ± 0.282
12	diAQA2	16.50	—	0.87 ± 0.004
13	diAQA3	17.33	2.07 ± 0.02	3.12 ± 0.033
14	Luteolin	20.40	—	0.47 ± 0.002
15	Nepetin	20.65	—	0.43 ± 0.001
16	Apigenin	23.07	—	0.37 ± 0.002
17	diAQA4	23.10	2.83 ± 0.044	under LOQ
18	Hispidulin	23.57	—	0.43 ± 0.001
19	Jaceosidin	24.07	—	0.51 ± 0.004
20	diAQA5	24.66	0.99 ± 0.011	0.14 ± 0.004
21	diAQA6	25.72	under LOQ	under LOQ
22	Cirsilineol	26.73	—	0.03 ± 0.004

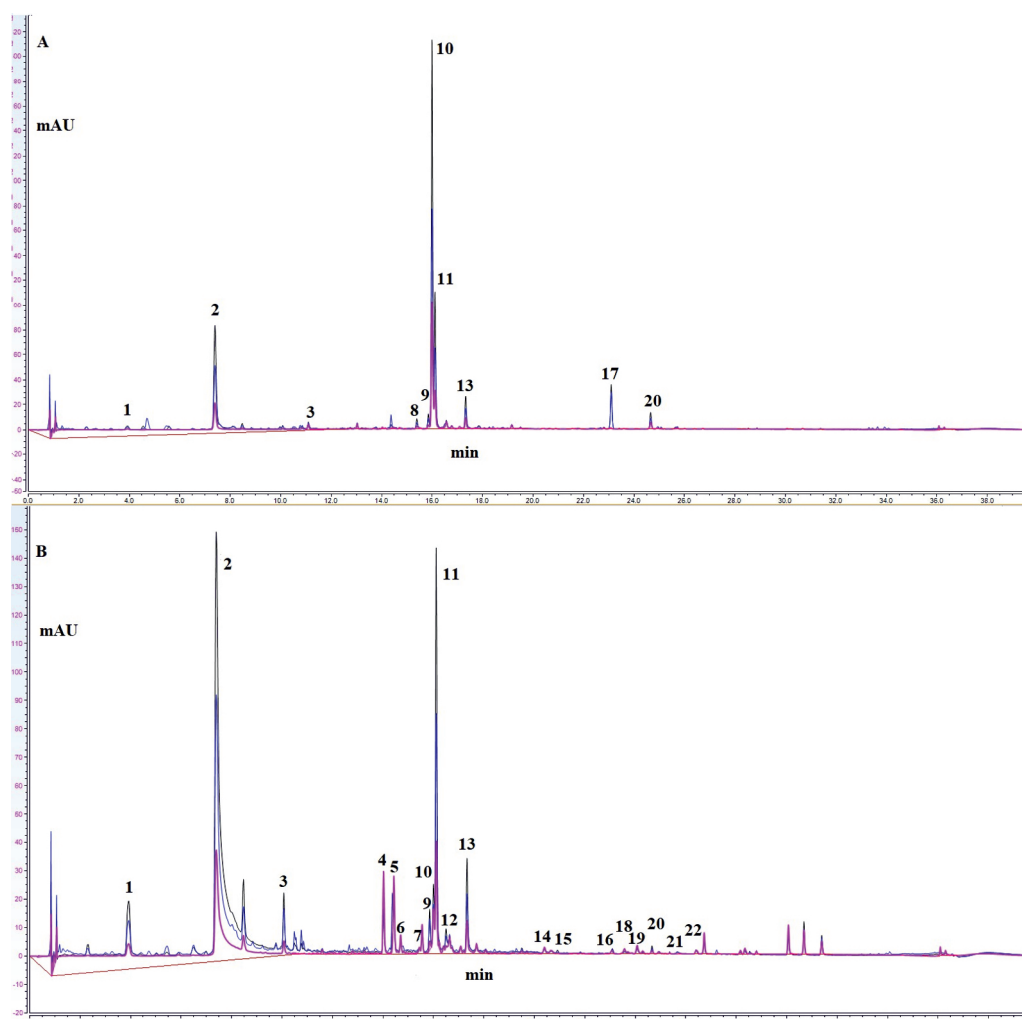


Fig. 1. UHPLC-DAD chromatograms of the studied *E. ritro* extracts; A – flowering heads; B – leaves; wavelengths: 310 nm, 280 nm, 350 nm. For the compounds numbering see Table 2

vealed moderate quantity of flavonoids isoquercitrin (4) and rutin (5), as well as different aglycones (14-16, 18, 19) (Table 2).

Enzyme inhibitory effect. The enzyme inhibitory potential of the studied *E. ritro* plant organs was determined against acetyl- and butyrylcholinesterase (AChE and BChE), tyrosinase, α -amylase, α -glucosidase and lipase (Table 3). The flowering heads revealed a pronounced activity against lipase (9.06 ± 1.43 mg OE/g) and α -amylase (0.37 ± 0.02 mmol ACAE/g). Conversely, the leaves showed high AChE and α -glucosidase inhibitory activity (2.33 ± 0.09 mg GALAE/g and 0.37 ± 0.01 mmol ACAE/g, respectively). BChE and tyrosinase inhibitory capacity is reported up to 1.41 ± 0.35 mg GALAE/g and 45.66 ± 0.84 mg KAE/g,

T a b l e 3

Enzyme inhibitory activities of the studied *E. ritro* extracts

Extracts	AChE	BChE	Tyrosinase
	inhibition	inhibition	inhibition
	(mg GALAE/g)	(mg GALAE/g)	(mg KAE/g)
Leaves	2.33 ± 0.09	1.03 ± 0.04	43.32 ± 2.21
Flowering heads	1.79 ± 0.03	1.41 ± 0.35	45.66 ± 0.84
Extracts	Amylase	Glucosidase	Lipase
	inhibition	inhibition	inhibition
	(mmol ACAE/g)	(mmol ACAE/g)	(mg OE/g)
Leaves	0.29 ± 0.01	0.37 ± 0.01	4.99 ± 0.60
Flowering heads	0.37 ± 0.02	0.23 ± 0.04	9.06 ± 1.43

Values are reported as mean ± SD of three parallel experiments. GALAE: Galantamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent; OE: Orlistat equivalent

respectively, both detected for the flowering heads extract. Thus, the leaves and mostly the flowering heads of *E. ritro* appear to have a prominent enzyme inhibitory activity towards the studied enzymes. The data for AChE and amylase are comparable to those announced by ZENGİN et al. [6], but our results for BChE are pronounced.

Lipase, glucosidase and amylase are key digestive enzymes and their inhibition is one of the most widely studied mechanisms applied to determine the potential efficacy of natural products as hypolipidemic and hypoglycaemic agents [15]. Therefore, the lipase inhibitory activity has been extensively used as an anti-obesity agent and it is successfully applied in the field of weight loss [16,17]. α -Glucosidase enzyme controls glucose level in blood and is responsible for diabetes [18]. Additionally, studying the tyrosinase inhibitory potential involved in melanogenesis could be practically used in the protection of the harmful consequences of sunlight [19]. Thus, the examined *E. ritro* extracts with the aforementioned capacity appear to have a health promoting usage towards these pathological conditions.

Cytotoxicity assay. Herein, to evaluate the *E. ritro* cytotoxicity, the human monocytic cell line (THP-1 cells), a classical immune model, was used (Fig. 2). After 24 h of incubation with flowering heads and leaves extracts, a slight rise of metabolic activity was measured between 2 and 200 $\mu\text{g.mL}^{-1}$. A toxicity of 60% for 1000 and 100% > 2000 $\mu\text{g.mL}^{-1}$ was reached for leaves extract. Regarding the flowering heads extract, 60% toxicity was observed at concentrations above 2000 $\mu\text{g.mL}^{-1}$. All of these toxic doses are very high and could be considered meaningless.

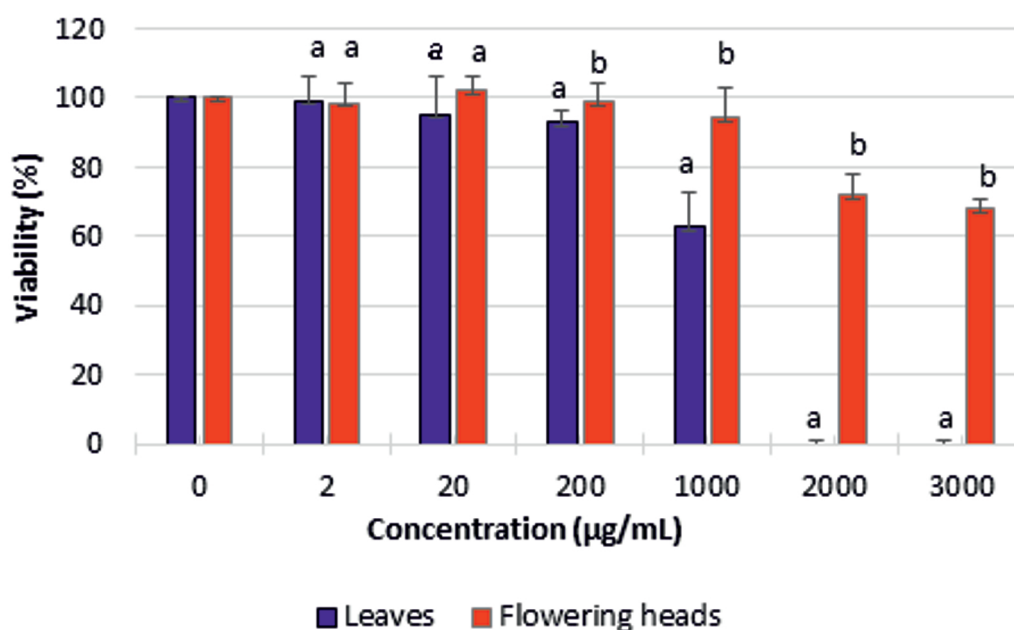


Fig. 2. Cytotoxicity of the studied *E. ritro* extracts towards THP-1 cells (one-way ANOVA, different letters indicate significant difference between plant parts in the same concentration (a and b), $p \leq 0.05$)

Our data for the anti-proliferative activity of the studied taxon are in line with the previously carried out investigation on other *Echinops* species [4].

Conclusion. The studied *Echinops ritro* leaves and flowering heads extracts presented noteworthy enzyme inhibitory properties and anti-proliferative activity, and could be assumed as a potential candidate with health benefit applications.

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Department of Pharmacognosy

**Department of Chemistry*

Faculty of Pharmacy

Medical University – Sofia

2 Dunav St

1000 Sofia, Bulgaria

e-mail: vbalabanova@pharmfac.mu-sofia.bg

dzheleva@pharmfac.mu-sofia.bg

rgevrenova@pharmfac.mu-sofia.bg

y_voynikov@pharmfac.mu-sofia.bg

***Physiology and Biochemistry*

Research Laboratory

Department of Biology

Science Faculty

Selcuk University

Campus, Konya 42130, Turkey

e-mail: gokhanzenengin@selcuk.edu.tr

****Institut Jean Lamour*

UMR CNRS 7198

Université de Lorraine

CNRS, IJL,

F-54000 Nancy, France

e-mail: olivier.joubert@univ-lorraine.fr