© 2016 IJSRST | Volume 2 | Issue 1 | Print ISSN: 2395-6011 | Online ISSN: 2395-602X
Themed Section: Science and Technology

Antimicrobial, Antioxidant and Anti-Inflammatory Activities of the extract of a Moroccan endemic Narcissus: *Narcissus broussonetii*

Amal Razik[1], Farida ADLY[1], Mina MOUSSAID[1,2], Chadi BERHAL[4], Hassan MOUSSAID[3], Abdelaziz ELAMRANI[*5], Touria Ould BELLAHCEN [7], Nourdinne BOURHIM[1], Jamal Jamal EDDINE[5], Carles CODINA[6] and Jaume BASTIDA[6], Mohamed LOTFI[1]

[1]Laboratory of Biochemistry, Cellular and Molecular Biology, Faculty of Sciences, Casablanca, Morocco
[2]Laboratory of Organic Synthesis and Biological Studies, Faculty of Sciences, Casablanca, Morocco
[3]Laboratory of Pharmaceutical Chemistry, Institute of Pharmacy, Brussels, Belgium
[4]Laboratory phytosanitary Faculty of Agriculture, Beirut, Lebanon
[5]Laboratory of synthesis, extraction, and Physico-chemical study of organic molecules, Faculty of Sciences Aîn-chock, Casablanca, Morocco
[6]Department of Natural Products, Plant Biology and Soil Science, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain
[7]Biology Department, Faculty of Sciences Ain-chock, Casablanca, Morocco

ABSTRACT

The antioxidant, antibacterial and anti-inflammatory activities, of the extract of the *Narcissus broussonetii* bulbs were studied. The report concerning those properties of this Moroccan endemic species is described for the first time. Nine main alkaloids compounds were identified by GC and GC-MS such as tazettine, pretazettine, homolycorine, lycorine, ismine, 3-epimacronine and papiramine. The extracts of *N. broussonetii* up to 40 mg/100g b.wt, revealed a significant anti-inflammatory effect in mice and showed also a significant scavenging activity at 10 mg mL^{-1}. However, the extract showed a moderate antibacterial activity against all the microorganisms tested.
Keywords: *Amaryllidaceae*, *Narcissus broussonetii*, Alkaloids, Pharmaceutical Activities.

I. INTRODUCTION

The Narcissus which belongs to the family of Amaryllidaceous is long-lived herbaceous plants. However the classifications of Angiosperm Phylogeny Group (APG) place them rather among Alliaceae, in the probable subfamily of Amaryllidoideae [1]. The main environments of Narcissus, are Morocco, Iberian Peninsula and nearby countries [2]. They are also occurring in the East to the Ukrainian Carpathian Mountains, in Asia Minor and in China and Japan [3].

The Narcissus are bulbous plants, with basal sheets and hollow stems, contracted in plate, but sometimes forming a rhizome, carrying of one in several yellow or white flowers [4].These flowers are remarkable by the presence of a crown stemming from the perigone, presented in section or in trumpet, and which contain six petalloids tetals, and six stamens [5,6]. The fruits are out of trefoil capsule containing generally numerous egg-shaped dry seeds, and often black. The roots are fleshy and contractile, usually persistent during the rest period, and an underground bulb, which sink profoundly into the ground [7, 8].

Narcissus plants are mostly toxic, contain numerous sorts with big variability of forms and colours; their strong smell can give the migraine, and also loosens a

slightly sweetened and peppered flavor. It is since known century for a medicinal custom, and used in perfume shop [3, 7].

The Narcissus broussonetii is an endemic species of Morocco which quite localised in the coastal region; It is a hardy perennial plant which passes by a period of biological dormancy; it flowered after the first rains (after October); his environment extends of Mohammedia (30 Km north of Casablanca) until the Oualidia and Eljadida (90 Km south of Casablanca); it lives in the small rocky hills with in fresh ground, and the rocky beds of the old rivers which thrown themselves into the Atlantic. It is the flat with white flowers and appears in little dense population from 10 to 60 cm in height, strong, rustic, but threatened in its territory; it multiplies by sowing or by division bulbs; the inhabitants know the plant by the strong toxicity for the cattle [9].

The alkaloids are the main secondary metabolite of Narcissus plants. They have an interesting therapeutic effect, a great diversity of pharmacological activities and are also toxic [10].

More than hundreds of alkaloids are known in the Narcissus species. They have apparently diverse chemical structures, but derive all of a single precursor, norbelladine. These poisons are studied with interest view they are biologically active and they are candidates to make drugs [11].

Recently, de Andrade et al (2012) studied the Moroccan Narcissus broussonetii and mentioned that twenty three alkaloids were identified, including the very rare dinitrogenous alkaloids obliquine, plicamine, and secoplicamine. Pretazettine, a potent cytotoxic alkaloid, was also isolated from N. broussonetii. The EtOAc extract of N. broussonetii showed notable antiprotozoal in vitro assay against Trypanosoma cruzi, with an IC50 value of 1.77 g/ml. [12]

The aim of this study is to evaluate the antioxidant, antibacterial and anti-inflammatory activities, of the ethanolic extract of the Narcissus broussonetii. The result of this investigation will allow us to examine the possibility to exploit those extracts as new pharmacologically acceptable drugs in the future.

II. METHODS AND MATERIAL

The mathematical editor on which along with text you can also write

2.1. Sampling

The bulbs of the Moroccan endemic *Narcissus broussonetii* were collected during November and December 2011 from the Atlantic region of Casablanca. The plant material was identified according to the flora of Morocco [13] and also by Pr. M. Rejdali, Agronomic Institute and Veterinary Hassan II, Rabat (Morocco) and confirmed by Pr Carles Codina, and Pr Jaume Bastida, Department of Natural Products, Plant Biology and Soil Science, Faculty of Pharmacy, Barcelona, Spain. A voucher specimen is deposited in the Herbarium of the Department of Botany and Ecology at the Agronomic Institute and Veterinary Hassan II, Rabat (Morocco), in our laboratory at the Chemistry Department at the Faculty of Science Ain-chock, Casablanca (Morocco) (herbarium n° Nb2912008) and also at the University of Barcelona Herbarium (BCN 58745). [21]- Jahandiz, E. and Maire R. 1934. Catalogue des plantes du Maroc. Tome III, imprimerie Minerva, Alger, Algérie

2.2. Preparation of the extracts

The extraction was performed following the protocol described by Labrana et al (12 bis). Bulbs of *Narcissus broussonetii* (3 kg) were crushed and macerated with EtOH for 48 h. The extract was evaporated under reduced pressure. The bulb crude extract (extract A) was (115 g). The residue dissolved in H_2O and acidified with 5% H_2SO_4 to pH 2-3. The acidic fraction was extracted with petroleum ether. After removing neutral material, the aqueous solution was extracted with ethyl acetate to provide ethyl acetate fraction (extract B). Basifying the aqueous solution up to pH 10-11 with 10% NH_4OH and extracting it with n-Hexane gave hexane fraction (extract C). (12 bis) J. Labra˜na, A.K. Machocho, V. Kricsfalusy, R. Brun, C. Codina, F. Viladomat, J. Bastida, Alkaloids from Narcissus angustifolius subsp. transcarpathicus (Amaryllidaceae), Phytochemistry 60 (2002) 847–852.

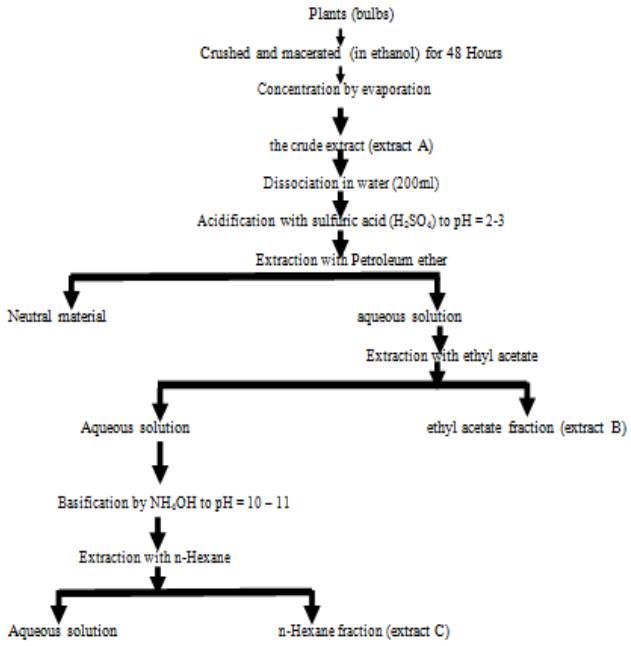


Figure 1 : General diagram of the method used for obtaining the fractions rich in alkaloids

GC–MS Analysis

The EI-MS spectra were obtained on an Agilent 6890NGC 5975 inert MSD operating in EI mode at 70 eV (AgilentTechnologies, Santa Clara, CA, USA). A DB-5 MS column (30 m×0.25 mm×0.25 _m, Agilent Technologies) was used. The temperature program was: 100–180 ◦C at 15 ◦C min−1, 1 min hold at 180 ◦C and 180–300 ◦C at 5 ◦C min−1 and 40 min hold at 300 ◦C.The injector temperature was 280 ◦C. The flow rate of carrier gas (helium) was 0.8 ml min−1. The split ratio was 1:20 for the analysis of the *N. broussonetii* extracts.

Identification of alkaloids by GC–MS

The alkaloids were identified by comparing their GC MS spectra and Kovats retention indices (RI) with our own library database. This library has been continually updated and reviewed with alkaloids repeatedly isolated by our group and identified using other spectroscopic techniques such as NMR, UV, CD and MS [13,14–15].

2.3. Evaluation of antioxidant activity (in vitro)

The antioxidant activity of the *N. broussonetii* extract was studied *in vitro* using the DPPH method. The activity of DPPH (1, 1-Diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, Steinheim, Germany) radical scavenging was investigated according to the method of Peiwu *et al*. [16]. In this method, a methanolic solution of DPPH (2.95 ml) was added to 50 µl sample of different concentrations of the extracts (10 to100 mg ml^{-1}) in disposable vial. UV spectra were recorded on a UV-vis spectrophotometer HP 8453. The absorbance was measured at 517 nm at regular intervals of 15 sec for 5 min. Ascorbic acid was used as a standard with concentration (0.1 M) [17].

$$\boldsymbol{Inhibition}(\%)(\boldsymbol{Reactive\ reaction\ rate}) = \frac{\boldsymbol{Abs.}(\boldsymbol{DPPH\ solution}) - \boldsymbol{Abs.}(\boldsymbol{sample})}{\boldsymbol{Abs.}(\boldsymbol{DPPH\ solution})} \times \mathbf{100}$$

2.4. Anti-Inflammatory activity Animals

Adult's mice of both sexes, weighing 25-28 g, were used in the experiments. The animals were allowed access to standard mouse chow and tap water. They were maintained in a controlled environment at 22-25 °C and 60±5 % relative humidity with a 12 h dark/light cycle and acclimatized for at least one week prior to use. The animals were randomly assigned to different experimental groups. each group kept in separate cage. All animal procedures were performed after approval from the Ethics Committee of the National Research Centre and in accordance with the recommendations for the proper care and use of laboratory animals.

Carrageenan-Induced Rat Paw Edema

The carrageenan was used to evaluate the anti-inflammatory properties of our ethanolic crude extract [18]; the mice were randomly assigned to treatment groups. The sterile carrageenan lambda (100 µL of a 1% solution in saline) was injected sub-planter into the right hind paw of the mice. The contralateral hind paw received the same volume of pure saline solution (0.9% NaCl) and served as a normal control (non-inflamed paw). Carrageenan caused visible redness and pronounced swelling that was well developed during 5 h and persisted for more than 48 h [19]. The hind footpad thickness was measured with a micrometer caliber [20, 21] before and after carrageenan injection at 1, 2, 3, 4 and 5 h. Three groups of six mice were used. The first group was administered saline solution (1ml) and served as control. The second group was tested by crude extract (20 and 40 mg/100 g b. wt. orally); the third group was

tested by indomethacin (2 mg/100 g body. wt.). The oral administration of the crude extract or indomethacin was given 1 h after carrageenan injection.

The mean paw volume was measured immediately before carrageenan injection (Vo) and then every hour (Vt) for five hours post administration of the crude extract and control [20]. The anti-inflammatory activity is expressed as percentage of reduction of the edema in the treated mice compared to control mice. The percentage edema and the percentage inhibition were calculated as follows:

$$\% \text{ edema (E)} = \frac{Vt - Vo}{Vo} \times 100$$

2.6. Antimicrobial Activity

Ethanolic crude extract and fractions were tested against *Candida albicans* ATCC 28367, *Fusarium solani* ATCC 36031, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 *Klebsiella pneumoniae* ATCC 23467 *Salmonella enteritidis* ATCC 13312 and *Streptococcus pneumoniae* ATCC 49619. Identity of the bacteria (Origin ATCC Gene Bank) used in this study was confirmed by Microbial Identification System in Biotechnology Application and Research Center at the National Institute of Hygiene, Rabat, Morocco.

The bacterial strains were cultured overnight at 37 ºC in Mueller-Hinton Agar (MHA), and the tow fungi *Candida albicans and Fusarium solani* were cultured overnight at 30 ºC in Sabouraud dextrose agar [22].

The antimicrobial activity was studied using the well diffusion method [23]. The degree of growth inhibition was evaluated after 48hr for bacteria and 12hr for fungi and compared with the growth inhibition results obtained from the controls (Tetracycline for bacteria and Nystatin for fungi).

2.7. Statistical Analysis

Data were expressed as Mean±SE of at least three independent experiments. The differences between control and treated groups were determined by one-way ANOVA followed by the least significant difference (LSD) [24].

III. RESULT AND DISCUSSION

3.1. Chemical composition of the extracts

The isolated compounds led to the identification of seven alkaloids (Table 1) which the most important alkaloids are: Tazettine with an abundance of approximately 20.46% and 34.10% of the total mixture respectively in the crude extract and ethyl acetate fraction. In addition we observed the presence of other alkaloids with abundance significantly, as Homolycorine, Lycorine, Ismine, Prettazetine, and 3- Epimacronine.

Table.1: Main alkaloids identified from *Narcissus broussonetii*

Alkaloids	**Crude extract**	**n-Hexane**	**Ethyl acetate**
Tazettine	20.46		34.10
Ismine	14.84		13.85
Homolycorine	18.6	29.21	
Lycorine			29.21
8-O Demetilhomolicorine	16.84		
3-Epimacronine	2.06		2.06
Trisferidine			
Papiramine		0.61	
Pretazettine		11.34	
Total	***72.8***	***41.16***	***79.22***

3.2. Pharmacological Results

3.2. 1. Antioxidant Activity (in vitro)

The antioxidant activity of ethanol extract of *N.broussonetii* was studied in vitro using DPPH method. The result of the kinetics of DPPH scavenging reaction of ascorbic acid and *N. broussonetii* ethanol extract at different concentrations is given in figure 1. It showed a strong scavenging activity just after 5 second and the % of inhibition obtained were 82.5, 79.3, 77.9, 73.6, 72.4, 65.2, 61.7, 62.5, 66.1 and 66.9, respectively for the concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg mL^{-1}. The values found for these samples were lower than those of the positive control ascorbic acid (89.8). The lower concentration (10 mg mL^{-1}) seems to be more active than the higher one as shown in Figure 1.

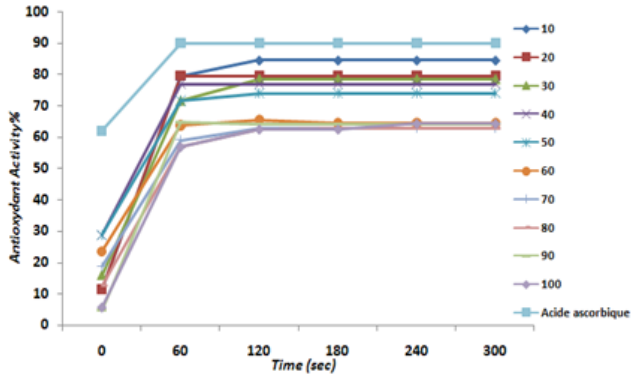
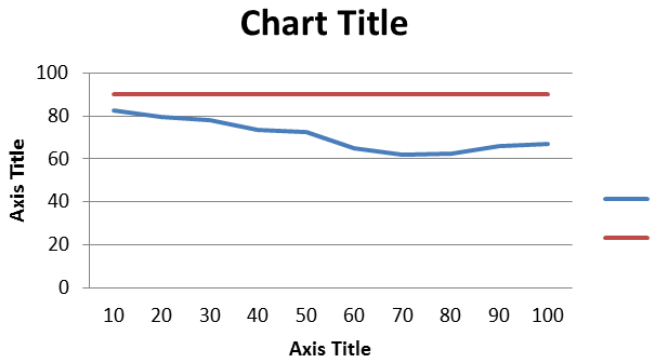


Figure 2 : The in vitro antioxidant activity of the ethanol extracts of *N. broussonetii*



3.2. 2. Anti-Inflammatory activity

Sub-planter injection of carrageenan into the mouse hind paw elicited an inflammation (swelling and erythma) and a time dependant increase in paw thickness compared with the pre carrageenan control value.

The anti-inflammatory activity is expressed as percentage of reduction of the edema in the treated mice compared to control mice.

The acute paw oedema response induced by sub-planter carrageenan was significantly reduced at 1, 2, 3, 4 and 5 h by 36.7, 32.6, 30.0 29.4 and 27.3% for 20 mg of *N. broussonetii* ethanolic extract and by 34.8, 38.0, 31.5, 32.9 and 30.4% for 40 mg of the same extract. For indomethacin, the % of oedema inhibition was 34.1, 44.6, 38.7, 48.1 and 49.2% respectively at 1, 2, 3, 4 and 5 h (table 2).

Table 2: Anti-inflammatory effect of *N.broussonetii* ethanolic extract at doses of 20 and 40 mg/100 g b. wt. on carrageenan-induced mice paw oedema

	After 1h		2h		3h		4h		5h	
groups	Oedema %	Oedema inhibition %	Oedema %	Oedema inhibition %	Oedema %	Oedema inhibition %	Oedema %	Oedema inhibition %	Oedema %	Oedema inhibition %
Control	67.1 ±5.6*	-	82.1 ±2.8*	-	85.6 ±3.6*	-	87.03 ±3.3*	-	92.4 ± 2.1*	-
N. broussonetii ethanolic extract (20 mg/100g b.wt)	42.1 ±3.4*	36.7	51.4 ±2.3*	32.6	58.8 ±3.7*	30.0	62.01 ±2.4*	29.4	61.5 ±2.3*	27.3
N. broussonetii ethanolic extract (40 mg/100g b.wt)	41.4 ±1.6*	34.8	45.5 ±5.3*	38.0,	58.5 ±4.0*	31.5	59.7 ±6.4*	32.9	58.5 ±4.5*	30,4
Indomethacin (2mg/100g b.wt)	44.2 ±3.1*	34.1	45.5 ±1.7*	44.6	52.5 ±2.5*	38.7	44.30 ±1.0*	48.1	43.6 ±3.4*	49.2

Data are presented as Mean±SEM; Significant change from control values at respective time; Points are denoted by *p<0.05 (one way ANOVA and Duncan test)

3.2.3. Antibacterial and antifungal activities

The result of antibacterial activity was summarized in table 3.

Table 3: Antimicrobial activity of ethanolic extract and fractions of *N. broussonetii*

Microorganisms	Zone of inhibition in mm*+ Extract and fraction			Standards
	Crude extract	Ethyl acetate fraction	n-Hexane fraction	
Fungi				Nystatin(50 IU)
Candida albicans	17	32	26	34
Fusarium solani	15	30	20	38
Bacterial				Tetracycline (30 IU)
Escherichia coli	16	28	20	24
Klebsiella. Pneumoniae	15	25	21	20
Salmonella enteritidis	10	24	19	25
Staphylococcus aurous	24	24	25	30
Streptococcus pneumoniae	18	22	22	26
Pseudomonas aeruginosa	17	23	21	<4

* Including the diameter of the well (4 mm); + Mean value of three independent experiments

The crude extracts showed significant activity against all Gram-positive bacteria except *Salmonella enteritidis*. The ethyl acetate fraction also showed moderate antibacterial activity against all bacteria whose zone of inhibition was ranging from (22-28 mm). The n-hexane fraction showed mild antibacterial activity against all Gram positive bacteria whose zone of inhibition was ranging from (19-21 mm).

The ethanolic crude extracts of *N. broussonetii* showed an activity in comparison to standard. Highest Zone of inhibition with ethanol extract of bulbs of *N. broussonetii* was found to be 24 mm against *Staphylococcus aurous* and lowest zone of inhibition was found to be 10 mm against Gram negative

Salmonella enteritidis. The n-hexane fraction showed highest activity against Gram negative *Staphylococcus aurous* 25 mm and lowest zone of inhibition was found to be 19 mm against Gram positive *Salmonella enteritidis*. The ethyl acetate fraction showed highest activity against Gram positive *Escherichia coli* 28 mm and lowest zone of inhibition was found to be 22 mm against Gram negative *Streptococcus pneumoniae.*

The antifungal screening was carried out against tow fungi *Candida albicans and Fusarium solani*. Ethyl acetate fraction was found to have a better antifungal activity (30-32 mm) than n-hexane (20-26 mm) and the crude extract (17-15mm).

IV. CONCLUSION

In this study, nine alkaloids from *Narcissus broussonetii* bulbs were reported. This investigation reports that the plant possesses antioxidant, anti-inflammatory and antimicrobial activities. Further studies are underway to reveal any additional potential of this plant as a biocide, and thus to determine the mechanisms of action.

V. ACKNOWLEDGEMENTS

This work was performed within the framework of project AECID A/011503/ and by the Transnational Cooperation Morocco-Spain.

VI. REFERENCES

[1] Angiosperm Phylogeny Group. 2003. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Bot. J. Linn. Soc., 141: 399-436.

[2] Fernandes, A. 1951. Sur la phylogénie des espèces du genre: Narcissus L. Bol. Soc. Brot. 25:148

[3] Meerow A.W., Fay M. F., Chase M.C., Guy C.L and Li Q-B. 2000. The New Phylogeny of the Amaryllidaceae. Herbertia., 54: 180-203.

[4] Lloyd D.G., Webb C.J., Dulberger R. 1990. Heterostyly in species of Narcissus (Amaryllidaceae), Hugonia (Linaceae) and other disputed cases. Plant Syst. Evol., 172: 215-227.

[5] Meerow A.W., Fay M. F., Guy C.L., Li Q-B., Q Zaman F and Chase M.C. 1999. Systematics of Amaryllidaceae based on cladistic analysis of plastid sequence data. Am. J. Bot., 86: 13-25.

[6] Worley A.C., Baker A.M., Thompson J.D and Barrett S.C.H. 2000. Floral display in Narcissus: variation in flower size and number at the species, population, and individual levels. Int. J. Plant Sci., 161: 69-79.

[7] Meerow A. W and Snijman D.A. 2001. Phylogeny of Amaryllidaceae tribe Amaryllideae based on nrDNA ITS sequences and morphology. Am. J. Bot., 88: 2321-2330.

[8] Dominicis RI., D'Amato G and Tucci GF. 2002. On the hybrid origin of Narcissus biflorus (Amaryllidaceae): analysis of C-banding and rDNA structure. Caryologia., 55(2).

[9] Fennane M. et Ibn Tattou M. 1998. Catalogue des plantes vasculaires rares, menacées ou endémiques du Maroc. Bocconea 8:193.

[10] Jennewein S and Croteau R. 2001. Taxol: biosynthesis, molecular genetics, and biotechnological applications. Appl. Microbiol. Biotechnol., 57: 13-19.

[11] Ingkaninan K., Irth H., Verpoorte R. 2002. Screening of Amaryllidaceae for biological activities: Acetylcholineesterase inhibitors in Narcissus. The genus Narcissus. G.R. Hanks (Editor) Harwood Publishers, London., 369-379.

[12] De Andrade J. P, Pigni N. B, Torras-Claveria L, Berkov S, Codina C, Viladomat F , Bastida J. 2012. Bioactive alkaloid extracts from Narcissus broussonetii: Mass spectral studies Journal of Pharmaceutical and Biomedical Analysis., 70: 13– 25

[13] Jahandiz, E. and Maire R. 1934. Catalogue des plantes du Maroc. Tome III, imprimerie Minerva, Alger, Algérie (12 bis) J. Labra˜na, A.K. Machocho, V. Kricsfalusy, R. Brun, C. Codina, F. Viladomat, J. Bastida, Alkaloids from Narcissus angustifolius subsp. transcarpathicus (Amaryllidaceae), Phytochemistry 60 (2002) 847–852.

[14] Bastida J., Lavilla R and Viladomat F. 2006. Chimical and biological aspects of Narcissus alkaloids, The Alkaloids: J. Chem and Biol., 63: 87–179.

[15] Viladomat F. Bastida J, Tribó G, Codina C, Rubiralta M. 1990. Alkaloids from Narcissus bicolor, Phytochemistry., 29: 1307–1310.

[16] Berkov S, Bastida J, Sidjimova B, Viladomat F, Codina C. 2008. Phytochemical differentiation of Galanthus nivalis and Galanthus elwesii (Amaryllidaceae): a case study, Biochem. Syst. Ecol., 36 : 638–645.

[17] Peiwu L., Hopia A., Jari S., Yrjnen T and Vuorela H. 1999. TLC method for evaluation of free radical scavenging activity of Rapeseed meal by video scanning technology. Proceedings of the 10th International Rapeseed Congress, Canberra, Australia.

[18] Govindaragan R., Rastogi S., Vijayakumar M., Shirwaikar A., Rawat A.K.S., Mehrotra S and Pushpangadan P. 2003. Studies on the antioxidant activities of Desmodium gangeticum. Biol. Pharm. Bull., 26: 1424-1427.

[19] Winter C.A., Risley E.A and Nuss G.W. 1962. Carrageenan induced edema in hind paw of the rat as an assay for anti-inflammatory drug. Proc. Soc. Exp. Biol. Med., 111: 544-547.

[20] Vinegar R., Truax J.F and Selph J.L. 1976. Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents. Eur. J. Pharmacol., 37: 23-30.

[21] Meng L., Mohan R., Kwok B.H.B., Elofsson M., Sin N and Crews C.M. 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proc. Natl. Acad. Sci., 96: 10403-10408.

[22] Obukowics M.G., Welsch D.J., Salsgiver W.J., Martin-Berger C.L and Chinn K.S. 2005. Novel, selective 6 or 5 fatty acid desaturase inhibitors as anti-inflammatory agents in mice. J. Pharmacol. Exp. Ther., 287: 157-166

[23] Chabbert Y.A., Daguet G. L. 1985. Techniques en bactériologie : antibiotiques en bactériologie médicale. Editeur: Flammarion, Médecine & Sciences, Sérologie bactérienne.

[24] Vanden Berghe D.A., Vlietinck A.J., 1991. Screening methods for antibacterial and antiviral agents from higher plants. In: Hostettmann K. Ed., Methods in plant biochemistry, vol. 6. London, UK, Academic Press, p. 47-69.

[25] Armitage P. 1971. Statistical Methods in Medical. Black Well Scientific Publications, London.F. Damerau, A technique for computer detection and correction of spelling errors. Communications of the ACM, 1964, Vol. 7, Issue 3, pp. 659-664.