NPC Natural Product Communications

2016
Vol. 11
No. 0
1 - 2

Chemical Composition, Antioxidant and Cytotoxic Activities of Essential Oil of the Inflorescence of *Anacamptis coriophora* subsp. *fragrans* (*Orchidaceae*) from Tunisia

Ridha El Mokni[a,b,c,d], Saoussen Hammami[e*], Stefano Dall'Acqua[f], Gregorio Peron[f], Khaled Faidi[e], Jeremy Phillip Braude[f], Houcine Sebei[b] and Mohamed Hédi El Aouni[a]

[a]*University of Carthage, Laboratory of Botany and Plant Ecology (SNA-214), Department of Life Sciences, Faculty of Sciences of Bizerte, Jarzouna, 7021, Bizerta, Tunisia*

[b]*University of Carthage, Laboratory of Soil Sciences and Environment, Mograne Graduate School of Agriculture, 1121 Mograne, Zaghouan, Tunisia*

[c]*University of Jendouba, Silvo-pastoral resources Laboratory, Silvo-Pastoral Institute of Tabarka, BP. 345, 8110-Tabarka, Tunisia*

[d]*IRESA, Laboratory of Forest Ecology, I.N.R.G.R.E.F, Tunis, Tunisia*

[e]*Research Unit 12-04, Applied Chemistry and Environment, Faculty of Sciences of Monastir, 5000, Monastir, Tunisia*

[f]*Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via F. Marzolo, 5, 35131 Padova, Italy.*

stefano.dallacqua@unipd.it

Received: February 27th, 2016; Accepted: XX, 2016

The chemical composition of the essential oil produced by steam distillation of the inflorescences of naturally growing *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. *fragrans* (Pollini) R. M. Bateman, Pridgeon & M. W. Chase (*Orchidaceae*) from Kroumiria, north-west Tunisia was studied by GC-MS, which led to the identification of 19 volatile components, representing 97% of the oil. The main constituents were methyl-(*E*)-*p*-methoxycinnamate (29.3%), 13-heptadecyn-1-ol (18.6%), 2,5-dimethoxybenzyl alcohol (14.1%) and 4-(1,1,3,3-tetramethylbutyl)-phenol (9.0%). DPPH radical scavenging revealed a weak antioxidant activity. In addition, the antiproliferative effects were evaluated on BxPC3 human pancreatic carcinoma cells and on 2008 human ovarian cancer cells showing significant effect. This is the first report of the chemical composition of essential oils obtained from *A. coriophora* subsp. *fragrans* inflorescences for North Africa. Further studies are needed to understand fully the possible mechanism of action behind the cytotoxic activity of the essential oil.

Keywords: Floral fragrance, GC-MS, *Anacamptis coriophora* subsp. *fragrans*, Orchidaceae

Orchids (*Orchidaceae*) are a group of plants with high diversity in color, size, bloom shapes and also in the way they smell [1,2]. They are considered nature's most extravagant group of flowering plants distributed throughout the world from the tropics to high alpine regions [3]. With 25,000 to 35,000 species [4], the *Orchidaceae* is regarded as the largest family in the plant kingdom [2,5,6]. Some of these species have aromatic inflorescences due to a distinctive flavor attributed to high concentration of volatile terpenic compounds serving mainly for the attraction of insects responsible for the dispersion of pollen and seeds [7]. Among these fragrant orchids, the genus *Anacamptis* Rich., comprised of 11 species and 25 taxa in 7 sections [8], is represented in Tunisia by 5 to 7 species [9,10]. The fragrant fug orchid, *Anacamptis coriophora* subsp. *fragrans* (syn. *Orchis fragrans* Pollini), is one of the rare fragrant plants naturally growing in Tunisia with wide distribution in the north of the country in moist sandy-clay soil habitats and dried puddles, ponds and marshes. It is a small plant (20 to 30 cm at most), the inflorescence of which is a fairly narrow oblong spike with a very pleasant smell of vanilla. Flowering occurs from April up to June (Figure S1 of supplementary data). Although reports on its distribution in the Mediterranean region (Europe and North Africa) show that it is a relatively common orchid [11-17], investigations of its essential oil components are still scarce. To our knowledge, nothing has previously been reported on the chemical composition of essential oils from inflorescences of orchids found either in Tunisia or in other parts of North Africa, and only two reports have been found regarding the chemical composition of flowers of *Anacamptis* species in Italy and France [1, 18-20]. Due to the scarcity of previously published data related to the volatiles of orchids, both in general and those restricted to the genus *Anacamptis*, and in continuation of our contribution to the chemical and biological studies of medicinal and fragrant plants growing spontaneously in Tunisia [21-23], the present paper reports novel information concerning the chemical composition of the volatile constituents of the essential oil from the inflorescences with seeds of *A. coriophora* subsp. *fragrans*, growing in Tunisia, using GC and GC-MS analysis.

Hydrodistillation of the inflorescences with mature seeds from *A. coriophora* subsp. *fragrans* gave a pale yellow colored, pleasant smelling oil. Nineteen compounds were identified and characterized by GC-MS, comprising about 97% of the whole constituents. The compounds identified are listed in Table 1 in elution order from the DB-1 GC column, along with the percentage composition of each component and Kovats indexes. The major compounds identified were methyl (*E*)-*p*-methoxycinnamate (29.3%), 13-heptadecyn-1-ol (18.6%), 2,5-dimethoxybenzyl alcohol (14.1%), 4-(1,1,3,3-tetramethylbutyl)-phenol (9.0%) and *p*-cresol (4.3%).

The chemical composition of the floral scent emitted by wild populations of *A. coriophora* collected in Italy and France have been previously reported [18,20]. In both reports, *p*-anisaldehyde

Table 1: Relative values (%) of odor compounds identified from the inflorescences of *Anacamptis coriophora* subsp. *fragrans* harvested from different wild populations in Kroumiria, north west Tunisia.

Compounds	R.T.*	KI**	%	Identification§
p-Cresol	8.4	1055	4.3	KI GC-MS ST
1-Undecyne	9.2	1081	0.9	KI GC-MS
p-Methoxyanisole	10.9	1127	1.1	KI GC-MS
Creosol	12.0	1158	0.3	KI GC-MS
3-Decyn-2-ol	12.8	1182	0.3	KI GC-MS
p-Anisaldehyde	13.5	1201	0.5	KI GC-MS ST
3,4-Dimethoxytoluene	13.6	1204	0.3	KI GC-MS
Thymol	16.0	1277	0.1	KI GC-MS ST
2,5-Dimethoxybenzyl alcohol	17.8	1328	14.1	KI GC-MS
Methyl cinnamate	18.1	1338	1.1	GC-MS ST
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	18.5	1351	0.7	KI GC-MS
E-2-Undecenal	19.4	1378	0.8	KI GC-MS
10-Dodecenol	19.7	1388	4.8	KI GC-MS
2-Dodecenal	20.1	1399	2.5	KI GC-MS
Methyl (*Z*) *p*-methoxycinnamate	24.2	1537	2.9	KI GC-MS
Methyl (*E*)-*p*-methoxycinnamate	26.4	1614	29.3	KI GC-MS
4-(1,1,3,3-Tetramethylbutyl)- phenol	28.0	1672	9.0	KI GC-MS
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	32.8	1859	5.7	KI GC-MS
13-Heptadecyn-1-ol	35.9	1990	18.6	KI GC-MS

*: Retention Time; **: Kovats index; §: ST: comparison with standard reference compound; KI: identification by Kovats index; GC-MS: identification by comparison with NIST 2012 MS database.

and *p*-dimethoxybenzene were the most abundant compounds [18,20]. The two samples differed in their total number of constituent; Salzmann *et al.* [18] reported 56 volatile compounds, of which 17 were identified, while Dormont *et al*. identified 27 constituents [20]. In addition, Dormont *et al*. [20] showed that the same natural fragrant fug orchid produced 21 volatile compounds (with purple colored flowers) and 23 volatile compounds (with white flowers). White morphs (specimens with white inflorescences) were found to emit greater amounts of volatile benzenoids, terpenoids, and fatty acid derivatives. The main volatile components for both morphs were *p*-anisaldehyde (38.4±18.5 - 38.5±10.8 %), 1,4-dimethoxybenzene (30.6±14.3 - 31.5±7.3%), methyl *p*-anisate (8.6±3.8 - 9.1±4.0%), 1,2,4-trimethoxybenzene (6.1±1.9 - 7.0±4.4%) and 1,2,4,5 tetramethoxybenzene (3.1±1.0 - 3.1±0.8%).

Our samples were obtained by hydrodistillation. Nineteen compounds were identified and the main components were different from those detected by SPME GC-MS analysis of floral emissions previously reported [18,20]. We also found *p*-anisaldehyde in our essential oil, but in very low amounts, while methyl-(*E*)-cinnamate was detected in our essential oil in higher amounts with respect to the literature [18,20]. These differences may be explained by the mode of extraction and sampling, the plant variability, as well as the environmental and climatic influences on the biosynthesis of volatile constituents.

A. coriophora subsp. *fragrans* essential oil demonstrated a weak antioxidant effect in the DPPH assay [26]. The concentration of extract required for scavenging 50% of the free radicals (IC_{50}) was approximately 1.3±0.1 mg/mL (Table 2).

Antiproliferative activity of the essential oil was assayed on BxPC3 cells (human pancreatic carcinoma cell line) and 2008 cells (human ovarian adenocarcinoma cell line). For comparison purposes, the cytotoxicity of cisplatin, one of the most commonly used chemotherapy drugs worldwide, was evaluated under the same experimental conditions. Dose-survival curves obtained after 72 h of drug treatment and the corresponding IC_{50} values are shown in Figure 1.

Table 2: IC_{50} concentrations of DPPH scavenging capacity of bioactive compounds.

Compounds	C (µg/mL)	Inhibition percentage	IC_{50} (mg/mL)
Essential oil of Tunisian *Anacamptis coriophora* subsp. *fragrans*	250	15.2±1.0	1.3±0.1
	500	20.2±1.0	
	1000	46.0±8.6	
	2000	73.7±2.0	
Quercetin	62	46.2±0.1	0.1±0.0
	125	89.6±0.2	
	250	90.0±0.7	
	500	92.6±0.2	
	1000	96.4±0.5	

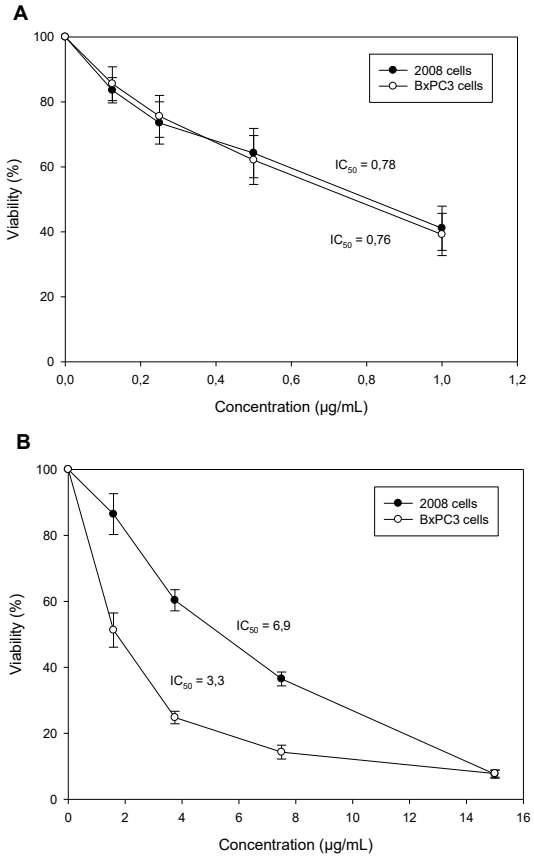

Figure 1: Cytotoxic activity against 2008 and BxPC3 cells. Cells ($3\text{-}5\times10^4$ mL^{-1}) were treated for 72 h with increasing concentrations of either essential oil (panel A) or cisplatin (panel B). IC_{50} values were calculated by the dose-response curves by means of four parameter logistic model ($p<0.05$).

In both cell lines the cytotoxic effect induced by the essential oil was dose-dependent, with mean IC_{50} values of 0.78 and 0.76 µg/mL against BxPC3 and 2008 cells, respectively. It is noteworthy that the IC_{50} values calculated for the essential oil were lower than those calculated for cisplatin (mean IC_{50} values of 6.9 and 3.3 µg/mL against BxPC3 and 2008 cells, respectively). However, further studies are needed to understand if this activity is due to a possible synergistic effect of the whole oil composition or could be related to the presence of the most abundant constituents.

A. coriophora subsp. *fragrans* is very similar to other species and to other plants of the closely related taxa within the *Orchis coriophora* group (*O. coriophora* subsp. *fragrans* (Pollini) Bateman, *O. coriophora* subsp. *martinii* (Timb.-Lagr.) Nyman (incl. *O. coriophora* var. *carpetana* Willk.) and *O. coriophora s. str*.) [13].

Significant differences in the chemical composition between Tunisian fragrant fug orchid essential oils and those from the northern shore of the Mediterranean Sea (Italy and France) were detected and might arise from several environmental (i.e. climatic, seasonal and geographical) and genetic differences or the diversity of pollinators [19]. In fact, *A. coriophora* produces nectar and is pollinated by various types of bees, including several bumblebees (*Bombus* spp.), honeybees (*Apis mellifera* Linnaeus, 1758, aggregate) and some moths of the genus *Zygaena* [27,28], and nectar production within the genus *Anacamptis* is probably a derived trait [29]. Variation in the inflorescences oil constituents may also be influenced by pollinator selection, which may have a direct impact on floral scent emissions on either side of the Mediterranean Sea. This hypothesis proposes a discrimination between chemotypes and argues in favor of a distinction between at least two major distant chemotypes: the first, North African (subsp. *fragrans* chemotype *africanus*), and the second, which includes European subspecies (subsp *fragrans* chemotype *europaeus*).

In conclusion, in the present investigations the chemical composition of the inflorescences of the fragrant fug orchid, *A. coriophora* subsp. *fragrans* from Tunisia was investigated. The essential oil was obtained by steam distillation, and its chemical composition was determined by GC and GC-MS. Among the 19 compounds identified, the main constituents were different to those reported in previously published papers, in which the chemical composition of the floral scent emitted by wild populations of *A. coriophora* collected in Italy and in France were studied. The *in vitro* antioxidant activity was studied showing weak effect. Furthermore, the cytotoxic activity on two tumor cell lines appeared to be surprisingly high, the calculated IC_{50} value being lower than those calculated for the reference chemotherapeutic agent cisplatin. Further investigations are required to clarify the mechanism of action behind this high cytotoxicity, and to study if the effect is due to the whole mixture of constituents or is related to specific compounds.

Experimental

Plant material and isolation of essential oil: The inflorescences with seeds of *Anacamptis coriophora* subsp. *fragrans* were collected in the Kroumiria region, northwest Tunisia, in April-May 2014. Harvested specimens were identified by Ridha El Mokni, one of the coauthors, botanist in the Laboratory of Botany and Plant Ecology, Faculty of Sciences of Bizerta, Jarzouna, Bizerta-Tunisia, where voucher specimens have been deposited. Dried material (about 200 g) was subjected to hydrodistillation for 3 h in a Clevenger type apparatus. The oils were dried over anhydrous sodium sulfate. The yield was $5x10^{-2}$ %.

GC-FID and GC-MS analysis: GC analysis of the oil was carried out on an Agilent 6840 N gas chromatograph equipped with a FID detector. The separation was achieved using a fused-silica-capillary column DB-1 (30 m × 0.25 mm i.d., 0.25 µm film thickness). The analysis was carried out in the following conditions: injector and detector temperatures, 210°C and 290°C, respectively; Helium was the carrier gas at a flow rate of 0.8 mL/min; split ratio 1:10; injection volume 1 µL. Temperature programming was 60°C with 3 min initial hold, and then 280°C at a rate of 3°C/min, and finally held isothermally for 5 min. GC-MS analyses were performed on a Varian 3900 gas chromatograph coupled with a Varian Saturn 2100IT ion trap mass spectrometer detector (MS), equipped with a an Agilent DB-5 fused-silica-capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Ionization mode was electronic impact at 70 eV. Mass range was set from 40 to 650 Da. Gas chromatographic conditions were the same as described for GC-FID. Components were identified by comparison of their mass spectra with those of the NIST Library (2012) and confirmed by comparing the retention indexes (relative to C6-C24 *n*-alkanes), and when available, comparing with authentic standards from Sigma-Aldrich (Milano, Italy). The percentage composition of the oil was calculated from the GC peak areas using the normalization method without correction factors. The data are reported as mean value of 4 oil injections.

Antioxidant activity using the DPPH radical scavenging assay: The antioxidant activity of the free *A. coriophora* subsp. *fragrans* bioactive compounds extracts was measured on the basis of the scavenging activity of the stable 1, 1-diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams *et al.* [24], with slight modifications. Keeping the volume constant, different concentrations of *A. coriophora* subsp. *fragrans* extracts (250, 500, 1000, 2000 µg/mL) were added to a methanol solution of DPPH (100 µM). Corresponding blank sample were prepared and quercetin (62-1000 µg/mL) was used as reference standard. The solution was left to sit in the dark for 30 min at room temperature and then absorbance was determined at 517 nm, using a UV-Vis spectrophotometer. Radical scavenging activity was calculated by the following formula:

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

Where A_c is the absorption of the control or blank sample (t= 0 min) A_s: is the absorption of the sample or test extract solution (t=30 min).

Antiproliferative assays: Cell cultures of human pancreatic carcinoma cells, BxPC3, were obtained from the American Type Culture Collection (ATCC, Rockville, MD), whereas human ovarian cancer cells, 2008, were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy). Cell lines were maintained in the logarithmic phase at 37°C in a 5% carbon dioxide atmosphere in RPMI-1640 medium (Euroclone, Milan, Italy) containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units·mL^{-1} penicillin and 50 µg·mL^{-1} streptomycin) and 2 mM *l*-glutamine.

The growth inhibitory effect towards human cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay [25]. Briefly, 5.103 cells/well were seeded in 96-well microplates in growth medium (100 µL) and then incubated at 37°C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 µL of a 5 mg·mL^{-1} MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and, after 5 h additional incubation, 100 µL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M was added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted *vs* drug concentration.

Supplementary data: A figure representing *Anacamptis coriophora* subsp. *fragrans* inflorescences is also available.

References

[1] Salzmann CC, Schiestl FP. (**2007**) Odour and colour polymorphism in the food deceptive orchid *Dactylorhiza romana*. *Plant Systems and Evolution*, ***267***, 37–45.

[2] Joshi G, Tewari LM, Lohani N, Upreti K, Jalal JS, Tewari G. (**2009**) Diversity of orchids on Uttarak hand and their conservation strategy with special reference to their medicinal importance. *Report and Opinion*, ***1***, 47-52.

[3] White KJ, Sharma B. (**2000**) Wild orchids in Nepal, the guide to the Himalayan orchids of the Tribhuvan Rajpath and Chitwan Jungle. White Lotus Press, Bangkok, Thailand.

[4] Cribb P, Govaerts R, Prat D. (**2005**) Just how many orchids are there? In *Proceedings of the 1^{8th} World Orchid Conference*, Raynal-Roques A, Roguenant A. (Eds). Turriers Naturalia Publications, Dijon, France.

[5] Dressler RL. (**1993**) *Phylogeny and classification of the orchid family*. Cambridge University Press, Cambridge,

[6] Hossain MM. (**2011**) Therapeutic orchids: traditional uses and recent advances-An overview. *Fitoterapia*, ***82***, 102-140.

[7] Pauli A. (**2006**) Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. *Molecular Research Review,* ***26***, 223-268.

[8] Kretzschmar H, Eccarius W, Dietrich H. (**2007**) *The orchid genera* Anacamptis, Orchis *and* Neotinea*, phylogeny, taxonomy, morphology, biology, distribution, ecology and hybridization*. Echino Media Verlag, Bürgel, Germany.

[9] Le Floc'h E, Boulos L, Vela E. (**2010**) *Catalogue synonymique commenté de la flore de Tunisie*. Banque Nationale de Gènes de la Tunisie, Tunis, Tunisia, pp. 360-362.

[10] Dobignard A, Chatelain C. (**2010**) *Index synonymique de la flore d'Afrique du nord.* Vol. 1. Éditions des conservatoires et jardin botaniques, Genève, pp. 171-172.

[11] Camus EG, Camus A. (**1921**) *Iconographie des Orchidées d'Europe et du Bassin Méditerranéen.* Paul Lechevalier, Paris, France.

[12] Maire R. (**1959**) *Flore de l'Afrique du Nord*. Paul Lechevalier, Paris, France, p. 396.

[13] Soó R. (**1980**) *Orchis* L. In *Flora Europaea*. Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA. (Eds) Cambridge University Press, Cambridge, ***5***, pp. 337-342.

[14] Delforge P. (**1994**) *Guide des Orchidées d'Europe, d'Afrique du Nord et du Proche-Orient*. Delachaux & Niestlé, Lausanne/Paris.

[15] De Belair G, Vela E, Boussouak R. (**2005**) Inventaire des orchidées de Numidie (N-E Algérie) sur vingt années. *Journal of European Orchids,* ***37***, 291-401.

[16] El Mokni R, Mahmoudi MR, Sebei H, El Aouni MH. (**2010**) Aperçu sur la diversité taxinomique et la distribution écologique de l'orchidoflore des régions de Kroumirie-Mogods (Tunisie septentrionale). *Actes du 15e colloque de la Société Française d'orchidophilie*. Société Française d'Orchidophilie, Montpellier, *7*, 66-72.

[17] El Mokni R, Vela E, El Aouni MH. (**2012**) Prospections orchidologiques dans les monts des Mogods et leurs environs (Tunisie septentrionale). *Journal Europäischer Orchideen*, ***44***, 365-380.

[18] Salzmann C, Cozzolino S, Schiestl F. (**2007**) Floral scent in food-deceptive orchids: species specificity and sources of variability. *Plant Biology*, ***9***, 720-729.

[19] Salzmann C, Nardella AM, Cozzolino S, Schiestl FP. (**2007**) Variability in floral scent in rewarding and deceptive orchids: the signature of pollinator-imposed selection. *Annals of Botany*, ***100***, 757-765.

[20] Dormont L, Delle Vedove R, Bessière JM, Schatz B. (**2014**) Floral scent emitted by white and coloured morphs in orchids. *Phytochemistry*, ***100***, 51-59.

[21] Benzarti A, Hammami S, Piras A, Falconieri D, El Mokni R, M'Henni MF. (**2013**) Effects of different ecological conditions and extraction techniques on the quality of volatile oils from flaxleaf fleabane (*Erigeron bonariensis* L.). *Journal of Medicinal Plant Research*, ***7***, 3059-3065.

[22] Faidi K, Hammami S, Ben Salem A, El Mokni R, Garrab M, Mastouri M, Gorcii M, Trabelsi Ayedi M, Taglialatela-Scafati O, Mighri Z. (**2014**) Polyphenol derivatives from bioactive butanol phase of the Tunisian narrow-leaved asphodel (*Asphodelus tenuifolius* Cav., *Asphodelaceae*), *Journal of Medicinal Plant Research*, ***8***, 550-557.

[23] Faidi K, El Mokni R, Joshi RK, Hammami S, M'Henni MF, Mighri Z. (**2014**) Comparative study on the chemical constituents of essential oils from different organs of the Sicilian *Kundmannia* (*Kundmannia sicula* L.) DC. (Apiaceae) growing spontaneously in Tunisia. *Natural Product Research*, ***28***, 1680-1684.

[24] Brand-Williams Wm, Cuvelier ME, Berset C. (**1995**) Use of free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft & Technologie*, ***28***, 25-30.

[25] Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. (**1988**) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Research*, ***48***, 589-601.

[26] Amarowicz R, Pegg R, Rahimi-Moghaddam P, Barl B, Weil J. (**2004**) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chemistry*, ***84***, 551-562.

[27] Van der Cingel NA. (**1995**) *An atlas of orchid pollination*. AA Balkema Publishers, Rotterdam, the Netherlands.

[28] Claessens J, Kleynen J. (**2011**) *The flower of the European orchid form and function*. Jean Claessens and Jacques Kleynen, Geulle, the Netherlands.

[29] Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, Chase MW. (**2003**) Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (*Orchidaceae*)*. Botanical Journal of the Linnaean Society*, ***142***, 1-40.