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Novel report of the phytochemical composition from *Fraxinus excelsior* pollen grains

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Summary

In this research, we investigated the phytochemical profiles for two models of aqueous (Aq) and methanolic (Me) pollen extracts of *F. excelsior* from three pollination periods from hermaphrodite flowers (H) of polygamous and male flowers of pure male (M) in order to identify their constituent compounds. Pollens of both phenotypes H and M were collected during three pollination periods and were analyzed by light and scanning electron microscopy (LM/SEM). The total phenolic content (TPC) and flavonoids content (TFC) was measured using Folin-Ciocalteu and aluminum chloride ($AlCl_3$) methods, respectively. Antioxidant activities were evaluated using ferric reducing antioxidant power (FRAP) and scavenging free radical $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. GC-FID and GC/MS were used to identify the chemical composition of essential oils. There was a significant difference ($P < 0.001$) between the means of TPC for M and TFC for the H. Comparison of H and M antioxidant activities showed that DPPH (IC_{50}) to be (2.977 ± 0.117 μM) during the second pollination period of M and (4.877 ± 0.021 μM) for first period of H. The majority of the compounds identified were linalool (35.42%) from the monoterpenoides in H and Delta-cadinene (43.22%) belonging to the sesquiterpenes in M. We concluded that there is a significant difference between the H and M compounds in pollen at different periods.

Key words: Antioxidant activity, Essential oils, *Fraxinus excelsior,* Oleaceae, Pollen grains

Introduction

Pollen grains or plant's male gametophytes are essential for plant reproduction (Nepi and Franchi, 2000). In recent decades, pollen has been utilized in melissopalynology as a nutrient source for bees, in traditional medicine as well as in food diets. Pollens contain secondary metabolites such as flavonoids, terpenoids and compounds with potential antioxidant activities such that some researchers have even referred to pollen as 'a valuable nutritional supplement' (Feás et al., 2012). Although pollen can act as an immune stimulant, it is one of the most common allergens and often cross-reacts with other allergens from different sources and geographical locations (D'Amato et al., 2007). Hence being a 'necessary evil', investigating pollen with respect to pollination periods, its ontogeny as well as determining its geographical distribution may help in adding knowledge on its potential applications (Jannesar et al., 2017).

Pollen dispersion from trees, weeds, and grasses during pollination periods is mostly carried out by wind (anemophilous pollens) or animals (entomophilous pollens) such as birds, bees, butterflies, etc. Some of these trees, especially *Fraxinus excelsior,* have been described as polygamous, trioecious and/or subdioecious and their pollination is carried out by both winds and insects. *F. excelsior* belongs to the Oleaceae family that is an important source of hardwood. Its distribution extends across Europe from the Atlantic coast in the west into continental Russia in the east through to the northern parts of Spain, Italy, Greece, and as far south as 37ºN in Iran (Nonotte-Varly, 2015; Wallander, 2008).

The genus *Fraxinus* exhibit great diversity within the same population such as being pure male, female and polygamous trees. The male flowers consist of two stamens, hermaphrodite flowers having stamens and one pistil. The female flower consists of more or less rudimentary stamens as well as one pistil. The genus *Fraxinus* is very diverse in terms of the morphology of its reproductive system and this diversity has led to the emergence of species with a high degree of adaptation to environmental conditions (Lee et al., 2012).

Research in herbal medicine has shown that the leaves, fruits, and seeds of the *Fraxinus* have some effective antiviral activity, anti-inflammatory, antioxidant activity and cytotoxic effects (Ayouni et al., 2016; Fernández-Manjarrés et al., 2006; M'sou et al., 2017). Chemical components including coumarins, secoiridoids, phenylethanoids, flavonoids, and lignans have been isolated from *Fraxinus* species. Also this plant is a source of food for honeybees, and may contribute some flavor in honey (Giovanetti and Aronne, 2011; Kostova and Iossifova, 2007).

In Iran, the *Fraxinus* genus in most parts of the country consists of two main species: *F. excelsior* and *F. angustifolia* (Kaveh et al., 2014). The aim of the present research is to investigate and analyze the extracts of H and M pollen grains of *F. excelsior* flowers and to compare the pollens during three periods of pollination with respect to antioxidant activities of Aq and Me extracts using three methods namely TPC, TFC and identification of the chemical composition of essential oils during the developmental stages of pollens, for purposes of evaluating their potential as natural anti-oxidant sources for food and phytotherapeutic products. In addition, we sought to note the stage of effective pollen grain production to optimize the isolation and purification processes.

Materials and methods

Plant material

F. excelsior H and M individual trees located around Tehran at latitudes and longitudes about of 35° 69′N and 51° 42′E were located (Taravat et al., 2017). *F. excelsior* pollen grains were collected during the three periods of pollination, from January 2015 to February (pollination period 1), February to March (pollination period 2) and March to early April 2015 (pollination period 3) resulting in six sample groups: H_1, H_2 and, H_3: pollen grains of hermaphrodite flowers and also, M_1, M_2 and, M_3: pollen grains of male flowers for three pollination times.

For each stage, 52 g of pollen grains have been collected. After confirming their herbarium code, Iran, Tehran, National Botanical Garden, 89122 AUH-Azadi et al., (Kaveh et al., 2014) H and M were separated based on their morphology and anatomy with the help of photomicrographs obtained using a stereomicroscope (Olympus, Japan).

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Anther and pollen grains anatomy

In order to examine pollen grains at their different stages of pollination, anther samples of H and M were collected during all developmental stages. Samples were fixed in FAA 70 (20% formaldehyde, 10% acetic acid, 70% ethanol, v/v), embedded in paraffin and sectioned to a thickness of 7-10 µm using a Micro DC 4055 microtome (Microteq, Germany). Staining was performed using Periodic Acid Schiff (PAS) protocol as suggested by CHEHRGANI and SEDAGHAT (2009), and contrasting was done using Meyer's Hematoxylin. Anthers at each developmental stage were observed and photographed under an Axiostar Plus light microscope (Zeiss, Germany). For each stage of H and M, at least twenty flower samples were studied and images were taken with a Coolpix 4500 digital camera (Nikon, Japan).

Purification and observation of pollen grains with Scanning Electron Microscopy (SEM)

The pollen grains from each sample of H and M were screened by sieving (70 mesh) and separated from the anther residues during the three different periods of pollination. To ensure a more than 90% purity, the pollen grains from each sample were verified for their ultrastructure using SEM. Pollen grains were dried prior to gold coating, then observed by the SEM microscopy XL 30, (Philips, Holland) and then scanned at 26kV (SHOUSHTARI et al., 2013). Collected samples were kept in 50 ml tubes at -20 °C for further analysis.

Extraction and sample preparation of *F. excelsior* pollen grains

10 g of the powdered *F. excelsior* pollen grains from each sample were dried in the shade and homogenized with a grinder before extraction. Samples were then extracted for 24 h with 100 ml Methanol for Me extract 1:10 w: v using a Soxhlet apparatus at room temperature. Aqueous (Aq) extracts were prepared by continuous shaking at room temperature (RAJA et al., 2016). The twelve extracts samples obtained for H and M pollens for the three pollination period were: H_1Aq, H_2Aq, H_3Aq, H_1Me, H_2Me, H_3Me, M_1Aq, M_2Aq, M_3Aq, M_1Me, M_2Me, and M_3Me. The extracts were concentrated in a rotary evaporator (Eyela OSB-2100, Japan) under a vacuum. Finally, the freeze-dried samples were in a freeze dryer (ALPHA 2-4, Martin Christ, Germany) for 8h and then kept in a refrigerator at 4 °C until further use.

Determination of total phenolic content (TPC)

TPC was quantified using the Folin-Ciocalteu method (RAJA et al., 2016). Briefly, 1 mg/ml of the pollen extract was mixed with 1 ml of 0.1 N Folin-Ciocalteu reagent (Sigma, USA). The mixture was kept at room temperature for 2-5 min, followed by addition of 1.0 ml of 20% Na_2CO_3. After 10 min of incubation at ambient temperature, the mixture was then centrifuged at 12,000 × g for 8 min. The absorbance of the supernatant was measured at 760 nm and the content of soluble phenolic compounds was determined using gallic acid (GAE) (Bio basic Canada), with (0-30 ppm) used as the standard. The standard curve was constructed using GAE in the range of 1.5-50 µg/ml. The TPC was calculated from the standard curve, $y = 0.0036x + 0.239$, $R^2 = 0.9938$ and TPC was expressed as mg GAE equivalents in 1 g of pollen sample (mg GAE/g).

Determination of total flavonoid content (TFC)

For total flavonoid content (TFC) determination, a modified spectrophotometric method was used (FREIRE et al., 2012). The stock solution was prepared from 5 mg of each sample with pollen extract being dissolved in 1 ml methanol. Next, 50 µl of $AlCl_3$ was added to the solution and mixed after incubation at room temperature for 15 min. The absorbance was then read with a spectrophotometer at 415 nm. The amount of TFC was determined using standard calibration curve of Rutin (Sigma Aldrich), in the range of 1.5-50 µg/ml^{-1}. The TFC was calculated from the standard curve, $y = 0.0004x + 0.551$, $R^2 = 0.9945$ and TFC were expressed as mg/ml rutin equivalents in 1 g of pollen grains (mg QE/g).

The ferric reducing/antioxidant power assay (FRAP)

The FRAP assay was performed as described by CHUA et al. (2013) and WATERHOUSE (2001) with minor modifications. This method depends on the reduction of a ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to (Fe^{2+}-TPTZ) at low pH. The FRAP reagent was prepared by mixing 10 mL of 300 mM L^{-1} acetate buffer pH 3.6 with 1 mL of 10 mM / LTPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM L^{-1} hydrochloride acid and 1 mL of 20 mM L^{-1} ferric chloride solution in distilled water. All solutions were used on the day of preparation. The samples were placed in 300 mL FRAP, 10 mL of each pollen extract sample and 30 mL deionized water, in order to obtain a final sample dilution of the reaction mixture of 1:34. The sample was incubated at 37 °C for 8 min and then absorbance was measured at 593 nm by a spectrophotometer against a blank. The relative activity of the samples was compared with standard ascorbic acid (2-10 µg/mL) used for constructing a calibration curve $y = -7.386x + 92.9$, $R^2 = 0.9973$).

Screening of the extracts for antioxidant free radical activity – DPPH

The samples were evaluated using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay with minor modifications (BERTONCELJ et al., 2007; RAJA et al., 2016). For each pollen extract, we used DPPH solution prepared at a concentration of 4×10^{-4} M in dimethyl sulfoxide (DMSO). 100 µL of each pollen extract and 100 µL of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated in the dark at room temperature for 1 h. The absorbance of the reaction mixtures was measured at 517 nm against a blank. The DPPH radical scavenging activity percentage inhibition was calculated as follows:

$$RSA\ (\%) = [1- (S - SB) / C] \times 100$$

where S is the absorbance of extract mixed with DPPH solution, SB is the absorbance of the same extract mixed with 100 µL DMSO, and C is the absorbance of control with particular solvent (without pollen extract). After obtaining the percentage of the radical scavenging antioxidant activity (RSA), the amount of IC50 of the extract and ascorbic acid were also determined. The RSA was expressed as IC50 (the concentration of the pollen extract samples (mg/ml), required to scavenge 50% of DPPH), calculated by a linear regression analysis and the calibration curve was $y = 0.001x + 0.049$, $R^2 = 0.925$.

$ABTS^{\cdot+}$ scavenging activity assay

The ABTS [2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], a green-blue radical cation ($ABTS^+$) solution was used to measure the scavenging activity of pollen extracts as described by (AYOUNI et al., 2016; RAJA et al., 2016). $ABTS^+$ was obtained by reacting 0.050 g ABTS salt with 0.008 potassium persulfate in 13mL deionized water. The solution was kept in dark for 16 h at room temperature. Prior to use, ABTS stock solution was diluted with PBS (4 mM, pH 7.4) until the absorbance of the solution reached 7.7 ± 0.02 at 734 nm. Twenty µL of samples solutions were added to 180 µL of this free radical solution and incubated for 6 min at room temperature. The absorbance of reaction mixtures was measured at 734 nm and the ascorbic acid calibration curve was used as the standard is expressed

in mM/g of dry extract using regression equation of calibration curve $y = -0.289x + 0.691$, $R^2 = 0.9991$. The percentage of RSA in this method was obtained using the following equation:

$$RSA\ (\%) = [(A0 - A1) / (Abs\ 0)] \times 100$$

Where A0 is the $ABTS^+$ absorbance value at the initial and A1 is the $ABTS^+$ absorbance value after 6 min of incubation. After obtaining the percentage of radical scavenging capacity (RSA), the IC50 value of extracts and ascorbic acid were determined.

Essential Oils extraction

Essential oils were extracted as described by FLAMINI et al. (2002), and SHARIF SHOSHTARI et al. (2012). 30 g of each pollen sample (H and M) from three different pollination periods were macerated in n-hexane solvent for 3 h to extract the oil components. The volatile oils were stored in dark glass bottles at 4 °C until analysis. The oil extraction percentage relative to yield weight of the pollen grains were in the range between 1.02 % and 2.33 % v/w, respectively (Tab. 5).

Gas Chromatography-Flame Ionisation Detector (GC-FID)

GC-FID analysis was performed using Shimadzu 15A gas chromatograph equipped with a split/split less (ratio 1:30), injector (250 °C) and a flame ionization detector (FID). The injector and detector (FID) temperatures were kept at 250 °C and 300 °C, respectively. Nitrogen was used as carrier gas (1 mL/min) and the capillary column used was DB-5 (50 m × 0.2 mm, film thickness 0.32 µm). The column temperature was kept at 60 °C for 3 min and then increased to 220 °C with a 5 °C/min rate and kept constant at 220 °C for 5 min. Relative percentage amounts were calculated from peak areas using a Shimadzu C-R4A chromatopac without the use of correction factors.

Gas Chromatography-Mass spectrometry (GC/MS)

GC-MS analysis was performed using a Hewlett-Packard 5973 gas chromatograph with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm). The column temperature was kept at 60 °C for 3 min and programmed to 220 °C at a rate of 5 °C/min and kept constant at 220 °C for 5 min. The flow rate of Helium as carrier gas was 1 mL/min; injection volume of 1 µL (1 % soln. in n-hexane). MS were taken at 70 eV, mass range, 30 to 350 amu and scan time, 2 scans/ sec. The components of the oil were identified by comparing their mass spectra with those of a computer library or with authentic compounds and confirmed by comparing their retention indices either with those of authentic compounds or with published data from the literature. The retention indices were calculated for all volatile constituents using a homologous series of n-alkanes (SHOSHTARI et al., 2012).

Statistical analysis

All experimental results were presented as mean ± standard deviation (n = 3) values for TPC, TFC and antioxidant activity. For comparison of pollination times, we used Repeated Measurement ANOVA (RM-ANOVA), Tukey's and Bonferroni tests were carried out using IBM, SPSS statistics 22.0. Independent sample t-test was used to compare means between Aq and Me extracts and H and M phenotypes. The relationship between which variables and antioxidant activity was analyzed using the correlation coefficients among those characteristics and Pair sample test was used for comparing antioxidant activities three methods. The difference with $p < 0.05$ was considered significant. All experiments were carried out in triplicate.

Results and discussion

Anther and pollen grains development

Both polygamous and male individuals' inflorescences panicles are classified under a group of inflorescence racemes (Fig. 1a). The floral ontogeny trend in inflorescence follows the initiation mechanism, meaning that basal flowers commence development with early initiation and then floral apex is observed in the next stages of growth. The difference between individual polygamous and pure male inflorescences is observable. Polygamous plants are associated with bisexual or hermaphrodite flowers, two stamens, bottle-shaped ovaries, and unisexual male flowers with two stamens (Fig. 1b). The results of this study regarding inflorescences models among male and polygamous trees are in compliance with previous studies such as WALLANDER (2008) and NAGHILOO et al. (2013), which reported on developmental stages of the inflorescence. The study of pollen grains' ontogeny showed that pollen mother cells (PMC) are large and typically occur clumped together near the tapetum. At this stage,

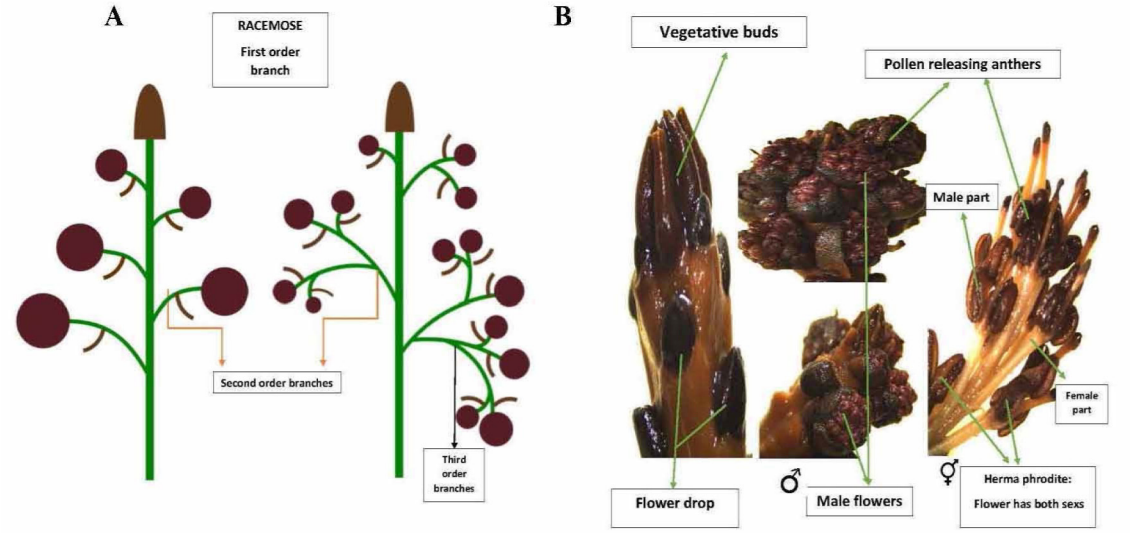

Fig. 1: A) The Inflorescences of *F. excelsior* are noticeable in hermaphrodite and pure male individual trees in both branching pattern and phyllotaxis. The pattern of flowers borne in tight lateral panicles or racemes. B) Hermaphrodite flowers of a raceme as opposed to male inflorescences panicles types.

PMC was well differentiated and is large in size with high density of cytoplasm. Each PMC is surrounded by an unevenly thickened layer of callose that is increased in size and forms a special wall that is callosic in nature (Fig. 2a-d). Then dyad stages and meiosis caused the formation of tetragonal tetrads are surrounded by a special callosic wall (Fig. 2e-h). During tetrads formation, taptums are differentiated mostly into binucleates or some in uninucleates (Fig. 2i-n). At the end of the tetrad stage, the callose surrounding the tetrads dissociates, and the four microspores separating from mature pollen grains have unicellular and bicellular stages in which the vegetative nuclear is bigger than the generative nuclear with both phenotypes being similar (Fig. 2m, n). Our results are partly in agreement with the finding of some prior reports about *Olea* reported by ZHU et al. (2013).

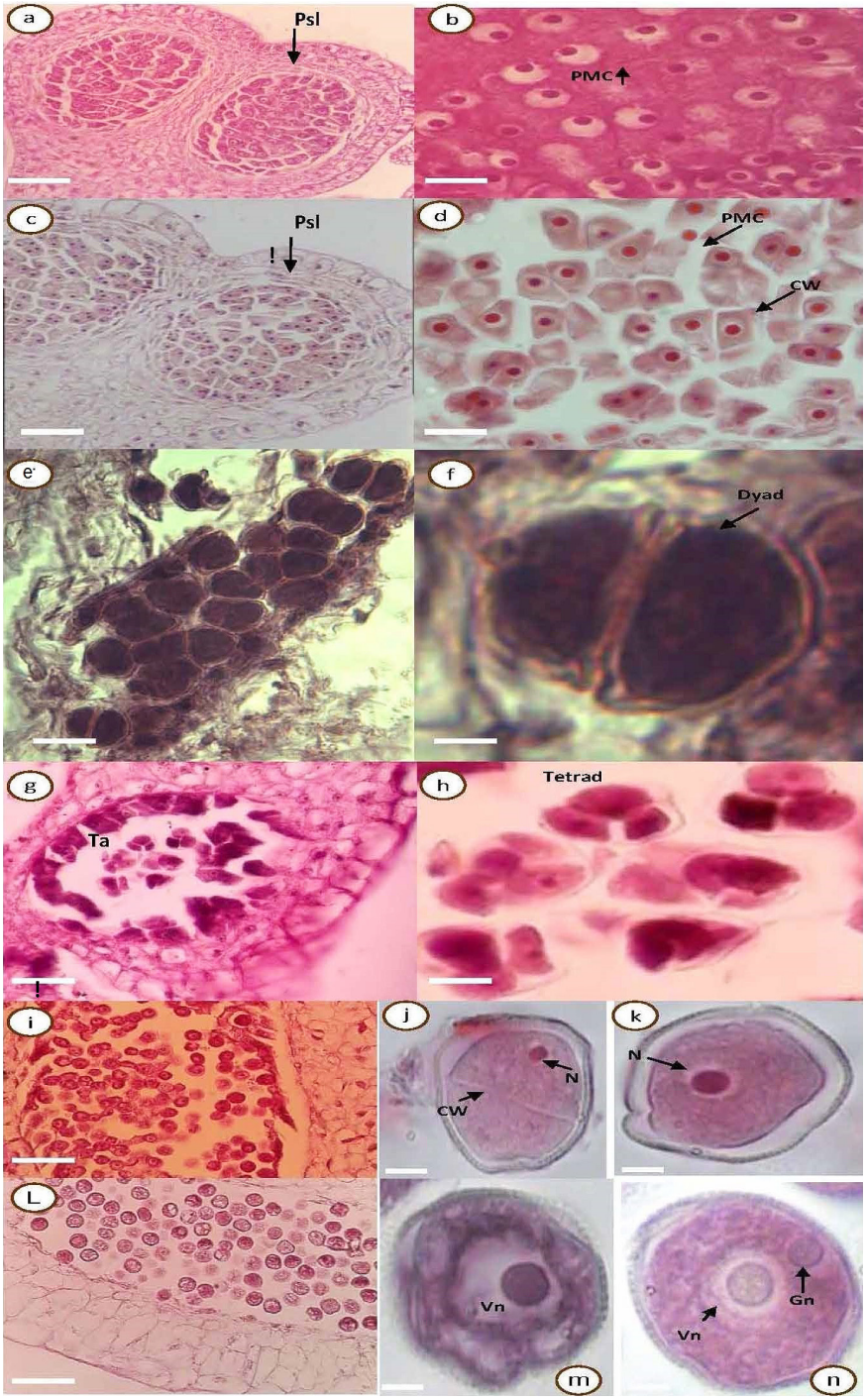

Fig. 2: Developmental stages of anther and pollen grains in *F. excelsior*. a) Cross section of a pollen sac containing pollen mother cells (PMC); b) Stages of PMC; c, d) the sections of anther shows two pollen sacs in pollen mother cells surrounded by a callosic wall (Cw); e, f) pollen sac in the dyad stages; g, h), tetrahedral tetrad. i-l) mononuclear microspores. m-p), mature pollen grains from different views a vegetative nucleus and two spermal nuclei. Abbreviations**:** psl, pollen sac layers; Ta, Tapetum layer; PMC, pollen mother cells; Cw, callosic wall; vn, vegetative nucleus; gn, generative nucleus; N, nuclei.
(a) Scale bar 50 µm, (b) Scale bar 15 µm, (c, d) Scale bars 50 and 15 µm, (e, f) Scale bars 25 and 8 µm, (g, h), Scale bar 20 and 8 µm, (i-l). Scale bars 15, 11and 6 µm (m-p), Scale bars 15, 11and 6 µm.

Purification of pollen grains

For phytochemical analysis, we purified pollen grains until the purity reached over 90%. The grains were then verified by SEM microscopy for H and M flowers (Fig. 3a, b). There were no morphological differences between M and H pollen grains in terms of form, structure and size. The shape of the prolate was circular in polar view. In mature H and M, the polar and equatorial axes were 18 mm and 15 mm respectively. The P/E ratio in both of them was ½. The type of pollens was tricolporate. The colpi were long with small circular end apertures (Fig. 3c). This observation was previously confirmed (Wallander, 2008; Sehr et al., 2009; Kaveh et al., 2014) while reporting on *F. excelsior*.

Total phenolic and flavonoid contents

Pollen extract yields obtained from 10 g of dry samples were measured for both of H and M phenotypes (Tab. 1). The highest yield was obtained from H_1 (34.50%) followed by H_2 (30.95%) and M_1 (30.14%). The TPC and TFC results of the tests are shown in (Tab. 2). Complementary methods are often used for analysis of samples for phenol and flavonoid contents. These methods measure the total phenol and flavonoid compounds on the basis of calorimetry. The results showed that the methanol extract was superior to the aqueous one (Fig. 4). The highest amount recorded was related to male individual methanolic extract which was found to be (105.880 ± 0.195 mg GAE/g) in the third period of pollination compared to H which had a value of (46.200 ± 1.997 mg GAE/g) at a significantly lower level $p < 0.001$. The highest amount of TFC was found to be related with the methanolic extract having a value of (163.733 ± 2.203mg rutin equivalent/g) in the H_2. But data in M_1 showed the lowest TFC (101.037 ± 1.970 mg rutin equivalent/g) On the other hand, the results obtained in relation to the amount of phenol and flavonoids from pollen grains showed a difference between H and M samples. The amount of TPC was higher in male pollens extracts and TFC were higher in hermaphrodites (Tab. 2).

An explanation for this might be the effect of epigenetics and the environment, which might ultimately have affected gene expression. However, from our point of view, carpels in pollen of H flowers could have acted as an inducing factor in accelerating the growth of pollens

Tab. 1: The yields of pollen extract samples after extraction and freeze-drying

Samples	Dry sample (g)	Yield (%)
H1	10	34.50
H2	10	30.95
H3	10	22.46
M1	10	30.14
M2	10	27.80
M3	10	21.75

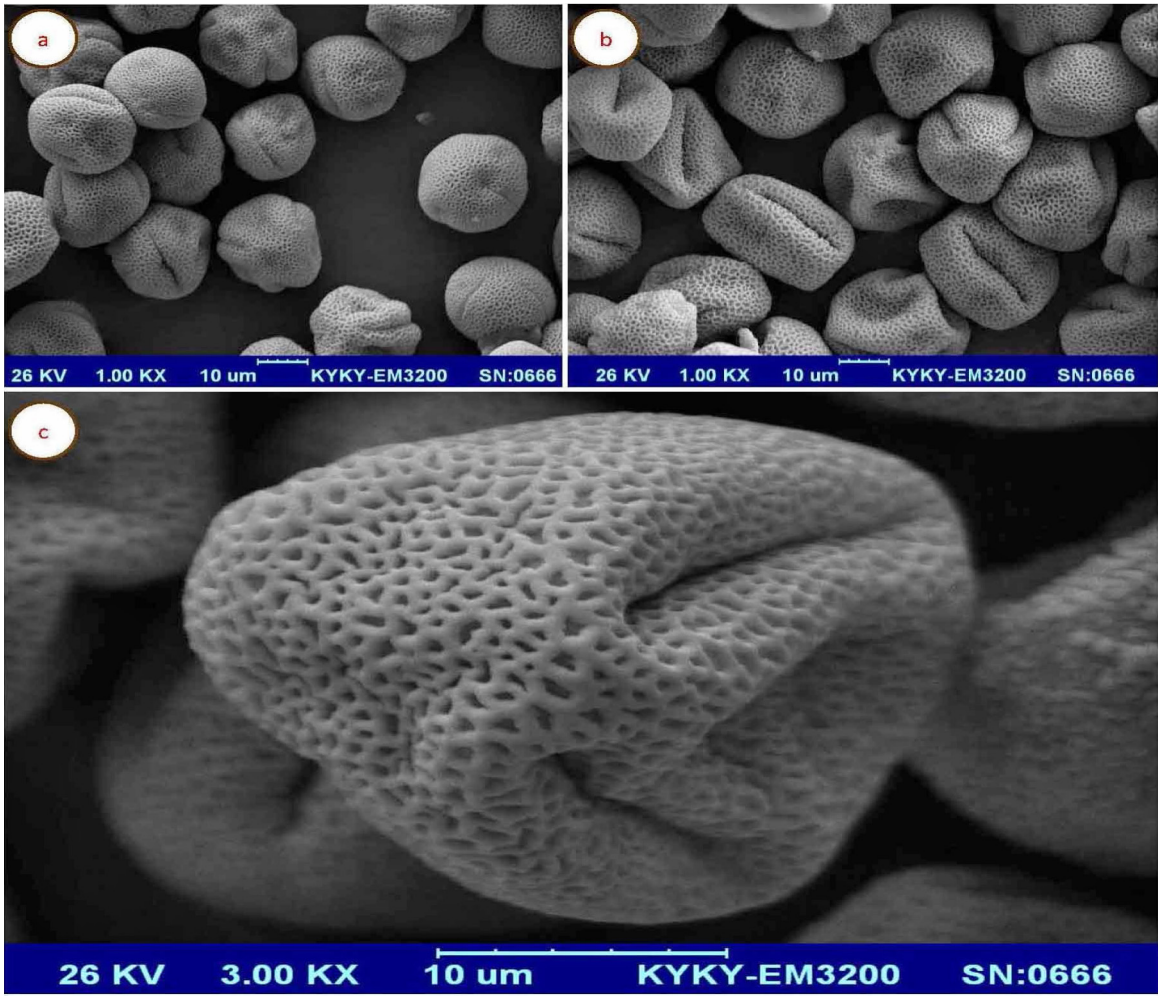

Fig. 3: The purity of both (H and M) pollen grains samples was checked by scanning electron microscopy (a, b). Scanning micrograph of a *F. excelsior* maturation in H and M phenotypes pollen grains *F. excelsior* mature pollen grain with three pores (c).

Tab. 2: Total phenol (TPC) and flavonoid (TFC) contents and antioxidant activities of Me and Aq extracts of two phenotypes of *F. excelsior* pollens with mean±SD triplicate measurement.
The content is represented as follows TPC as mg GAE g^{-1} DW; TFC as mg Rutin g^{-1} DW; FRAP as mg TE g^{-1} DW; DPPH as % inhibition.

	Time of pollination	H			M			P-value[2]	P-value[3]
		Aq	Me	P-value[1]	Aq	Me	P-value[1]		
		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD			
TPC	1	19.213 ± 0.595	29.173 ± 0.337	<0.001	42.623 ± 0.204	74.480 ± 0.674	<0.001	<0.001	<0.001
	2	24.350 ± 0.212*	32.773 ± 1.484	0.009	72.123 ± 0.071*	90.707 ± 0.369*	<0.001	<0.001	<0.001
	3	30.863 ± 0.221*+	46.200 ± 1.997*+	0.005	90.110 ± 0.053*+	105.880 ± 0.195*+	<0.001	<0.001	<0.001
TFC	1	96.683 ± 5.870	151.373 ± 2.106	<0.001	60.247 ± 0.189	101.037 ± 1.970	<0.001	0.008	<0.001
	2	81.600 ± 0.431	163.733 ± 2.203*	<0.001	45.210 ± 0.305*	81.667 ± 5.670	0.008	<0.001	<0.001
	3	67.783 ± 1.053+	121.120 ± 0.987*+	<0.001	23.487 ± 0.529*+	54.250 ± 0.577*+	<0.001	<0.001	<0.001
FRAP	1	0.933 ± 0.049	1.760 ± 0.013	<0.001	0.008 ± 0.001	1.320 ± 0.006	<0.001	0.001	<0.001
	2	1.027 ± 0.076	1.866 ± 0.019	<0.001	0.094 ± 0.004*	1.053 ± 0.040*	<0.001	0.002	<0.001
	3	1.087 ± 0.042	1.893 ± 0.003*	0.001	0.790 ± 0.199	1.386 ± 0.005*+	0.035	0.118	<0.001
DPPH (IC50)	1	6.220 ± 0.125	4.877 ± 0.021	<0.001	4.813 ± 0.169	4.083 ± 0.060	0.002	<0.001	<0.001
	2	8.367 ± 0.452*	9.043 ± 0.067*	0.119	2.977 ± 0.117*	3.140 ± 0.056*	0.094	<0.001	<0.001
	3	12.400 ± 0.292*+	10.807 ± 0.304+	0.003	5.497 ± 0.462*+	6.417 ± 0.268*+	0.041	<0.001	<0.001
ABTS	1	20.397 ± 0.466	39.710 ± 1.516	<0.001	30.700 ± 2.858	45.740 ± 0.567	0.010	0.022	0.003
	2	33.903 ± 0.761*	88.273 ± 0.204*	<0.001	29.950 ± 1.984	48.023 ± 0.032*	0.004	0.032	<0.001
	3	46.823 ± 0.172*+	94.447 ± 4.072*	<0.001	36.757 ± 0.743	56.207 ± 0.721*	<0.001	0.001	<0.001

P-value[1]: comparison between Aq and Me, P-value[2]: comparison between H and M (Aq extract), P-value[3]: comparison between H and M (Me extract)
*: in comparison with time 1, + : in comparison with time 2

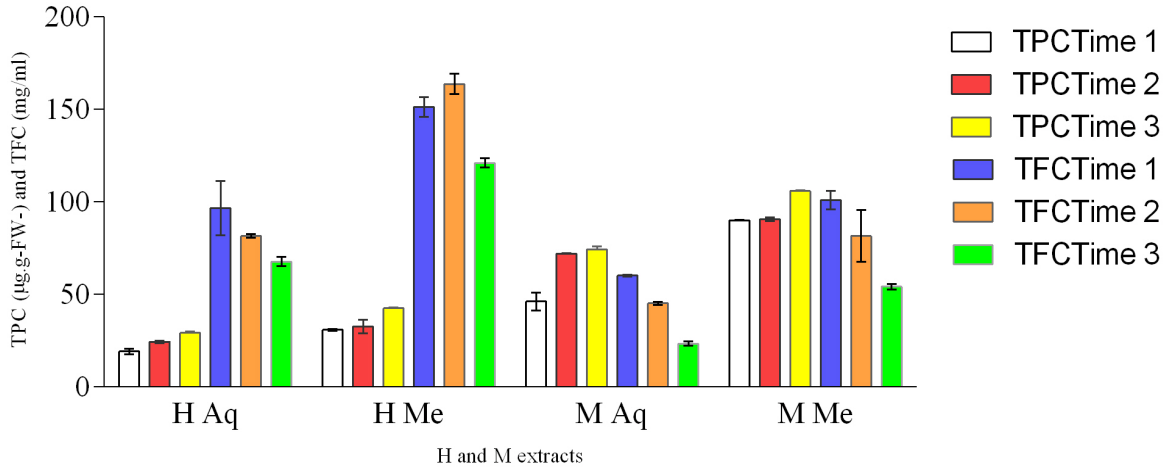

Fig. 4: Comparison of Aq and Me from 12 extracts in TPC and TFC, samples tests.

and their biosynthesis there by helping in the success of fertilization. Thus, the observed higher flavonoid content could be due to this. In addition, in male individuals, the phenol content was higher maybe because they are distributed on higher altitude hence access to air, UV radiation and exposure to sunlight, probably a kind of pollen protection against environmental stress conditions. The presence of phenol compounds in pollen grains has been reported in apiculture research by (RZEPECKA-STOJKO et al., 2015) and (HARIF FADZILAH et al., 2017). Structurally, pollen grains are capable of being digested in the stomachs of animals and humans, thus providing some antioxidant properties. However, the effect of their phytoestrogens is dependent on the structure of their molecules in different types of pollen. Plant phytoestrogens are used for phytotherapy and can inhibit the growth of certain cancer cells. Some pollen grains are a good source of flavonoids and phenols. In previous studies, analysis of TPC and TFC in pollen showed positive effects in animal models. However, due to the allergenicity of pollens, they must be carefully selected during their extraction (NONOTTE-VARLY, 2015). For this purpose, the present study was also aimed at carefully examining the different stages of pollination, to determine the point at which they contain maximum phenols and flavonoids.

Antioxidant activity

Three methods of assaying antioxidant activity were used in this study. Firstly, the FRAP method; a redox reaction associated with color change when Fe^{3+} are reduced to Fe^{2+}in mg TE g^{-1} DW (Tab. 2). Ascorbic acid equivalent antioxidant content (AEAC) used as the standard for FRAP was equivalent to (14.77 ± 0.42) of Fe^{2+} mg TE g^{-1} DW. The results of FRAP tests for 12 extracts did not vary significantly from their DPPH and ABTS scavenging activities. In this research, extracts from the H and M in pollination period 3

possessed the highest ferric reducing capacity (1.893 ± 0.003 and 1.36 ± 0.005 mg TE/g extract for H_3 and M_3, respectively), while the lowest capacity was exhibited by extracts from pollination period 1 (0.933 ± 0.049 and 0.008 ± 0.001 mg TE/g) extract for H_1 and M_1, respectively. These results were in agreement with those of MCHAREK et al. (2016) who reported the strong antioxidant activity of *Citrus limon* extracts, with the Me extracts having a stronger antioxidant activity compared with Aq extracts ones.

In the second method, we used the DPPH to investigate, the radical scavenging activities with ascorbic acid equivalent to (8.10 ± 0.001 µg/ml) being used as the standard for DPPH. The IC_{50} values obtained for the H and M pollen extraction during the three pollination periods are shown in Tab. 2 (HU et al., 2015).

In the third method, we investigated the antioxidant effects using the ABTS method. Using ascorbic acid (17.8 ± 0.01 µg/ml) as the standard. Analysis of extracts for antioxidant activities using FRAP, DPPH, and ABTS chemical methods showed that all the three methods offered comparable results. However, even though most chemical methods are simple and fast, results obtained did not depict a good correlation between them (Tab. 3). Some phenols compounds, for example, might require around 30 minutes for them to be fully analyzable but FRAP test is not able to do that. In addition, since all phenol compounds and flavonoids are pH sensitive, to obtain more accurate results, neutral pH should be maintained when using FRAP, 4 to 8 and 1 to 8 when using DPPH and ABTS respectively. Most phenol compounds in various plant organs, such as phenolic acid and flavonoids, have been linked with ranging pH changes. Scavenging antioxidants are one of the most important type of antioxidants to be evaluated for their effects as anti-allergic, anti-inflammatory, anti-asthma, antifungal, antibacterial and anti-cancer (HABEGGER and SCHNITZLER, 2012; HARIF FADZILAH et al., 2017). One of the advantages of DPPH is that, it typically takes 20-30 minutes to perform, which is preferable compared to the FRAP method. In addition, DPPH is a reversible method of assaying antioxidant activity (RZEPECKA-STOJKO et al., 2015). However, DPPH is affected by environmental conditions such as light, oxygen, and ambient pH, which affect the absorption and the chemical structure of the antioxidants. Similar to scavenging activity, total antioxidant activity of these bioactive components were found to be dose dependent, which is supported by the studies of Vuong and Mcharek (MCHAREK and HANCHI, 2017; VUONG et al., 2013). For the correlation of the results between the three tests given in Tab. 3, the ABTS method was shown to correlate well with the other tests. Our results for the comparative analysis by the pair statistical test methods are shown in Tab. 4. These results confirm that all three methods of antioxidant activity we suggested should be performed together.

Tab. 3: Pearson correlation between three methods of determining antioxidant activity

Pearson Correlation	FRAP	DPPH	ABTS
FRAP	1	0.466	0.724**
DPPH	0.466	1	0.534
ABTS	0.724**	0.534	1

**Correlation is significant at the 0.01 level (2-tailed).

Essential oils chemical composition

The yield of the pollen collected from both H and M samples was calculated on the basis of dry weight from the different stages of pollination as shown in Tab. 5. Based on the information in this table, the amount of essential oils varied, between 1.02 to 2.33 v/w (Tab. 5). The highest percentages of essential oils were related to the third stages of H pollination period and the lowest in the third stages of M pollination time. The results of separation and identification of components of essential oils, along with their percentages and KI, are shown in Tab. 6.

In investigating the chemical composition of essential oils in their first, second and third stages of pollination, we were able to identify forty-three components in H and thirty in M. It was also observed that the highest yield of H essential oils combinations was found to be than M phase. The highest constituents of H_1 compounds were, T-cadinol (19.95%), linalool (18.14%), camphene (14.87%), H_2, linalool (35.42%), camphene (13.24%), silane (6.92%), H_3, linalool (29.84%), alpha-terpinenyl acetate (13.67%), sabinene (8.13%), and for M_1, decane (24%), hexacosane (18.04%), tetracosane (12.70%), M_2, 1-methyl (28.44%), benzene acetic acid (19.88%), cyclotetrasiloxane (16.08%), M_3, delta-cadinene (43.22%), t-muurolol (18.74%), alpha-muurolene (9.89%). From this study, the percentage of essential oils at different stages of plant development ranged from 1.02% to 2.33%. M'SOU et al. (2017) reported about the leaves of *Fraxinus dimorpha* identified 40 components of essential oils from the aerial part of the plant (M'SOU et al., 2017). Another study on *Ligustrum delavayanum* identified a total of 28 components (BARÓNIKOVÁ et al., 2001) and essential oils research in leaves of *Jasminum subtriplinerve* also identified

Tab. 5: Essential oils content relative to pollen grain yield.

Samples	Essential oils percentage relative to yield weight of the pollens
Hermaphrodite flowers/ pollen grains pollination period 1	1.40%
Hermaphrodite flowers/ pollen grains pollination period 2	2.08%
Hermaphrodite flowers/ pollen grains pollination period 3	2.33%
Male flowers/ pollen grains pollination period 1	1.11%
Male flowers/ pollen grains pollination period 2	1.65%
Male flowers/ pollen grains pollination period 3	1.02%

Tab. 4: Paired sample tests for comparative three methods of antioxidant activity

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	FRAP - DPPH	-5.45194	2.77399	0.80078	-7.21445	-3.68944	-6.808	11	0.000
Pair 2	FRAP - ABTS	-46.47611	22.18798	6.40512	-60.57368	-32.37854	-7.256	11	0.000
Pair 3	DPPH - ABTS	-41.02417	21.17543	6.11282	-54.47839	-27.56994	-6.711	11	0.000

Tab. 6: GC-MS analysis of *F. excelsior* two phenotypes H and M for pollen grains essential oil compounds during three periods of pollination.

No.	Compounds	KI*	H1 %	H2 %	H3 %	M1 %	M2 %	M3 %	Methods of identification
Monoterpene Hydrocarbons									
1	α-pinene	936	0.90	2.98	2.27	4.87	_	_	MS-KI
2	Sabinene	973	2.32	2.88	0.58	_	_	1.03	MS-KI
3	Thymol	1290	3.63	_	0.40	_	_	_	MS-KI
4	Camphene	946	14.87	13.24	_	_	_	_	MS-KI
5	α-terpinene	1014	_	0.80	_	_	_	_	MS-KI
6	γ-terpinene	1054	0.90	1.14	29.84	_	_	_	MS-KI
7	β-pinene	974	_	2.40	8.13	_	_	_	MS-KI
8	β-thujene	971	_	_	_	_	_	2.69	MS-KI
9	(E) β-ocimene	1037	_	_	_	_	_	2.49	MS-KI
	Total identified (%)		**22.62**	**23.44**	**41.22**	**4.87**	_	**6.21**	
Oxygenated Monoterpenes									
1	P-Menth-1-en-9-al	1190	0.60	_	_	_	_	_	MS-KI
2	Linalool	1086	18.14	35.42	4.26	_	_	1.62	MS-KI
3	Linalylpropanoate	1173	1.33	_	_	_	_	_	MS-KI
4	Geranyl acetone	1430	0.99	0.90	5.38	_	_	_	MS-KI
5	α-Terpinenyl acetate	1335	_	_	1.33	_	_	_	MS-KI
6	Terpineol-4	1164	_	4.12	0.60	_	_	_	MS-KI
7	Bornyl acetate	1282	_	1.02	0.66	_	_	_	MS-KI
8	α-terpineol	1176	_	_	2.17	_	_	_	MS-KI
9	β- Terpineol	1137	_	1.91	_	_	6.19	_	MS-KI
10	Perilla alcohol	1280	_	_	4.65	_	_	_	MS-KI
	Total identified (%)		**21.06**	**43.37**	**19.05**	_	**6.19**	**1.62**	
Cyclic Monoterpenes									
1	α-Phellandrene	1002	0.40	_	0.47	_	_	_	MS-KI
2	β-phellandrene	1030	_	1.07	_	_	_	_	MS-KI
	Total identified (%)		**0.40**	**1.07**	**0.47**	_	_	_	
Sesquiterpene Hydrocarbons									
1	β-caryophyllene	1413	0.50	_	_	_	_	_	MS-KI
2	α-copaene	1379	_	_	0.40	_	_	_	MS-KI
3	Germacrene-D	1481	0.70	_	_	_	_	_	MS-KI
4	Calarene	1437	3.22	_	_	_	_	_	MS-KI
5	Caryophyllene Oxide	1582	0.98	_	0.20	_	_	_	MS-KI
6	γ-cadinene	1514	1.14	_	_	_	_	11.01	MS-KI
7	β-cubebene	1389	_	_	_	_	_	5.67	MS-KI
8	α-muurolene	1496	_	_	_	_	_	9.89	MS-KI
9	δ-cadinene	1520	2.84	_	_	_	_	43.22	MS-KI
	Total identified (%)		**9.38**	_	**0.60**	_	_	**69.79**	
Oxygenated Sesquiterpenes									
1	β-eudesmol	1641	_	_	0.50	_	_	_	MS-KI
2	T-cadinol	1640	19.95	_	_	_	_	_	MS-KI
3	Spathulenol	1572	2.54	_	_	_	_	1.09	MS-KI
4	T-muurolol	1633	_	_	_	_	_	18.74	MS-KI
	Total identified (%)		**22.49**	_	**0.50**	_	_	**19.83**	
Other Compounds									
1	Hexadecane	1600	_	_	_	4.69	_	_	MS-KI
2	Eicosane	2000	_	_	_	8.86	_	_	MS-KI
3	Octadecane	1800	_	_	_	3.07	_	_	MS-KI
4	Morphinan	2335	_	_	_	1.83	_	_	MS-KI
5	Hexacosane	2600	_	_	_	18.04	_	_	MS-KI
6	Tetracosane	2400	_	_	_	15.70	_	_	MS-KI
7	cyclotrisiloxane	3190	_	_	_	_	7.51	_	MS-KI
8	Cyclotetrasiloxane	1405	_	_	_	_	16.08	_	MS-KI
9	Benzeneacetic acid, 3,4-dimethoxy-, methyl ester	1625	_	_	_	_	28.44	_	MS-KI
10	Benzeneacetic acid, 3,4-dihydroxy	1827	_	_	_	_	19.88	_	MS-KI

No.	Compounds	KI*	H1 %	H2 %	H3 %	M1 %	M2 %	M3 %	Methods of identification
11	Tetracosamethyl-cyclododecasiloxane	2338	_	_	_	_	13.66	_	MS-KI
12	Cyclohexasiloxane, dodecamethyl	1363	_	_	0.90	_	_	_	MS-KI
13	Methyl palmitate	1901	_	_	0.47	_	_	_	MS-KI
14	Octane	800	_	_	_	3.01	_	_	MS-KI
15	Decane	998	_	_	_	24.00	_	_	MS-KI
16	Dodecane	1200	_	_	_	6.79	_	_	MS-KI
17	Tetradecane	1400	_	_	_	5.48	_	_	MS-KI
18	Palmitic acid	1959	_	1.36	_	_	3.17	_	MS-KI
19	4-Methylcyclohexanone	929	3.12	_	_	_	_	_	MS-KI
20	cis-Anethole	1230	0.90	_	_	_	_	_	MS-KI
21	cyclohexene	1409	0.60	_	_	_	_	_	MS-KI
22	Methyl eugenol	1369	3.50	1.91	13.67	_	_	_	MS-KI
23	Benzene	1157	_	1.13	_	_	_	_	MS-KI
24	Vanadium, oxotris(tri-methylsilanolato)-, (T-4)-	1301	_	_	_	_	1.90	_	MS-KI
25	Phenol	980	_	_	0.50	2.17	_	_	MS-KI
26	Silane	491	_	6.92	0.25	_	_	_	MS-KI
27	Octadecenal	2012	_	3.23	_	_	_	_	MS-KI
28	Trans-anethole	1262	_	_	5.92	_	_	_	MS-KI
29	Tetradecenal	1696	_	4.25	_	_	_	_	MS-KI
	Total identified (%)		**8.12**	**18.80**	**21.71**	**93.64**	**90.64**	_	

*KI: (Kovats index) measured relative to *n*-alkanes (C9-C28) on the non-polar DB-1 column under condition listed in the experimental section.

38 components (Dai et al., 2016). However, little information existed on the Oleaceae pollen grain oil. The chemical composition of *F. excelsior* pollen grains in two phenotypes (H and M) was found to differ from other Oleaceae species. We did not find any reference to this in the literature and this might be probably the first attempt in this regard. This may have been attributed by the different compounds in H and M pollens *F. excelsior* phytochemicals. In addition, results showed that two important monoterpenes, linalool and camphene being the major constituents of *F. excelsior* H pollens are being tried in clinical phase trials for drug development for their ability to inhibit the proliferation cancerous cells and to induce cell apoptosis (44). Linalool has been shown in several studies to exhibit some suppressive and cytotoxic effects on a melanotic melanoma C32 cells (Tundis et al., 2009), human leukemia, lymphoma cell lines, adenocarcinoma cells (Loizzo et al., 2008). Assessment of the effects of Camphene on some cancer cell lines showed some cytotoxic activity as well (Sobral et al., 2014). Also, two valuable compounds from *F. excelsior* M pollens, the sesquiterpens compounds such as delta-cadinene and t-muurolol had immunomodulatory potential and anti-cancer effects as reported (Krifa et al., 2015). But we suggested care to be used in extracting sesquiterpens compounds because according to Lundh et al. (2007) one of the factors creating contact dermatitis (Lundh et al., 2007). Factors such as the nature of the chemotype, ecology, climate and environmental conditions in various points of analysis may have contributed to the composition of the different phytochemicals obtained from herbal plants such as Oleaceae family. This is in agreement with Barbosa et al. and Pandey et al. (Barbosa et al., 2017; Pandey et al., 2014) that several factors such as mechanical, developmental stages or biological injuries such as herbivory, may influence the yield and chemical composition of essential oils. Seasonal variation and environmental factors such as light and relative humidity can also have large effects on the yield and composition of essential oils. These observations on the composition of essential oils are important for researchers in phytotherapy and aromatherapy for better extraction of pollens.

Conclusion

The results of the present study suggest that some essential oils possess strong medicinal activities, which can be utilized to treat certain diseases such as anti-inflammatory and anti-cancer ones. The role and effects of pollen derived antioxidant activity has been taken into consideration by most biological researchers in recent years. Extensive antioxidant compounds are available in plants but it is difficult to identify and isolate them from pollen grains as a source of food. In this research, we pursued three goals: firstly, comparing of pollen grains in different phenotypic forms H and M, secondly, the comparison and quantitation of pollens in plant's different pollination periods and thirdly, the comparison of the type of compounds extracted from Me or Aq. Precision in the periods for selection of pollen grains, as well as extraction can help researchers to optimize and standardize extracts, such as antioxidants and flavonoid compounds from pollens. Also, the phytochemical study of pollen compounds helps to obtain more accurate information about the internal contents of pollen grains and their interactions with pollen proteins. Furthermore, it is more likely to be used as a complementary method to investigate allergenicity of pollen proteins and to be more accurately reported.

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