Pollen grain flavonoid studies of four Iranian *Trifolium* (*Leguminosae*) taxa as future honey biomarkers

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Abstract. Pollen flavonoid studies provide useful information for evaluation of the nutritional and medicinal quality of natural honeys. In this study, pollen flavonoids of four Iranian *Trifolium* taxa are reported for the first time. Results have shown that all four studied taxa contain rutin, flavonoid sulphates, flavone *C* and *C-/O*-glycosides in their pollen. Morin is reported for the first time in the pollen of all studied taxa, with the exception of *T. tumens*. Apigenin, kaempferol, loteulin, myricetin, tricin, and vitexin were absent in the pollen of all studied species, but the pollen of *T. fragiferum* had quercetin. Flower pollen flavonoids are identical to those of the corresponding bee pollen confirming their use as chemical marker so important in ecology, pollination, honey qualification and preventing its adulteration.

Key words: chromatography, flavonoid, Iran, pollen grain, *Trifolium*

Introduction

Flavonoids are a set of polyphenolic compounds among the secondary metabolites in the different plant organs that are active especially in medicinal plants, which have pharmacological effect and contribute to human health (Estevinho & al. 2008). Flavonoids engage in a wide range of biological activities and also excise medicinal and pharmacological effect. In plants, flavonoids play an important role in auxin transportation, pollination, and modulation of reactive oxygen species. Furthermore, they have antibacterial, antifungal, antiviral, and anticancer properties (Parr & Bolwell 2000; Noori 2012). For two decades, flavonoids have been also used as biomarkers to fingerprint the floral origin of honey. Hence, various methods have been developed for determination of flavonoids and botanical classification of honey by means of chromatographical methods such as high-performance liquid chromatography (HPLC). Flower pollen flavonoids are identical to those of the corresponding bee pollen thus confirming their use as chemical markers. They are important because of their contribution to the color, taste and flavor of honey (Tomás-Barberán & al. 2001). Pollen collected by honeybees can be used as a dietary supplement for humans and their phenolics may have antioxidant effect (Campos & al. 2003; Leja & al. 2007; Rozema & al. 2001). Also, another important parameter of honey is its color, which reflects the floral source (Bertoncelj & al. 2007). Some studies have shown positive correlation between floral color intensity, flavones, flavonols, and phenolic content (Pontis & al. 2013).

Leguminosae species are especially rich in flavonoids, producing about 28 % of all known flavonoids and 95 % of all isoflavonoid aglycones (Hegnauer & Grayer-Barkmeijer 1993). About 850 compounds, including 362 isoflavones, that usually occur in a free state, are known in *Leguminosae* (Dewick 1993) and are obtained from *Papilionoideae* tribes and the species' organs (Ingham 1983). *Trifolium* (clover or trefoil) of *Fabaceae* (*Leguminosae*), *Faboideae* (*Papilionoideae*) and *Trifolieae* tribe is important in terms of its agricultural value and its species number (about 300) (Zohary & Heler 1984).

There are some studies on flavonoids in angiosperm pollen grain that support the use of flavonoid analysis for determination of the botanical origin of bee pollen (Tomás-Lorente & al. 1992). Alessandra & al. (2010) isolated for first time three flavonoids from the aerial parts of *Medicago littoralis* Rhode. Ceska & al. (1984) changed to Ceska & Derek (1984) isolated flavonol glycosides of quercetin, isorhamnetin and kaempferol from *Zea mays* pollen. Tomás-Lorente & al. (1992) isolated and identified 12 flavonoid glycosides and four aglycones from *Cistus ladanifer* bee pollen. Truchado & al. (2008) recently reported eight rhamnose and hexose glycosides of kaempferol from *Robinia pseudoacacia* honey. Studies of *Prunus amygdalus*, *Cistus* sp., *Echium* sp. and *Chrysanthemum* sp. bee pollen by TLC, 2D-PC and HPLC techniques have shown characteristic flavonoid patterns, which allow their use as biochemical markers of the plant origin. Furthermore, the flavonoid patterns of *Prunus amygdalus* and *Cistus* sp. pollen have appeared identical to those of the corresponding bee pollen confirming their use as chemical markers (Weston & al. 2000). Two flavonol glycosides were isolated from the methanolic extract of *Cannabis sativa* L. pollen grain, namely kaempferol 3-*O*-sophoroside and quercetin 3-*O*-sophoroside, by spectroscopic methods including high-field two-dimensional NMR experiments (Ross & al. 2005). Pollen flavonoid studies can provide useful information for the evaluation of nutritional and medicinal quality of natural honeys. These help classify the natural honeys and choose a specific honey for a specific health-promoting effect. In this study, pollen flavonoids of four Iranian *Trifolium* L. (*Leguminoseae*) taxa in aqueous-ethanolic extracts are investigated and reported.

Material and methods

Collection of plant material and preparation of pollen grains

Mature fresh flowers of four *Trifolium* taxa (*T. fragiferum* L., *T. repens* L., *T. repens* var. *repens* and *T. tumens* Steven ex M. Bieb.) were collected from Markazi Province, Iran in 2014, as described in Table 1. Plants were identified using the available references (Heller 1984; Haerinasab & Rahiminejad 2012). Specimens of each sample were prepared as herbarium vouchers and deposited at the Arak University Herbarium. Then, the pollen grain samples from anthers were air-dried after dissection of the flowers for flavonoid detection and identification.

Table 1. Information on collection and 2-Dimentional Paper Chromatography pollen flavonoids data of studied *Trifolium* taxa from Iran.

Voucher data	Taxon	Latitude (N)	Longitude (E)	Altitude (m)	Flavonoid type: Total flavonoids number	Flavonoid sulphates number	Flavone *C* and *C*-/*O*-glucosides number	Aglycones number
	Trifolium							
*CAM_1	*T. fragiferum*	33°54'	49°34'	2075	4	3	1	0
CAM_2	*T. repens*	33°47'	49°14'	2037	7	5	2	0
CAM_3	*T. repens*	33°54'	49°34'	2075	6	4	2	0
CAM_4	*T. repens* var *repens*	34°03'	49°38'	5919	4	2	2	0
CAM_5	*T. tumens*	33°53'	49°28'	1900	6	4	1	1

***CAM**: Mehrangiz Akbari Legumes Collection Number.

Extraction of the pollen grains

For a comparative analysis of the flavonoids, small extracts of all accessions were prepared by boiling 200 mg of powdered pollen grains for 2 min in 5 ml of 70 % EtOH. The mixture was cooled and left to extract for 24 h. The extract was then filtered, evaporated to dryness by rotary evaporation at 40°, and taken up in 2 ml of 80 % MeOH for analysis by 2-Dimensional Paper Chromatography (2-D PC).

Flavonoid analysis by 2-Dimensional Paper Chromatography (2-D PC)

For detection of flavonoids, *ca* 20 µl of each extract was applied to the corner of a quarter sheet of Whatman No 1 chromatography paper as a concentrat-

ed spot (10 applications of 2µl). The chromatogram for each sample was developed in BAW (n-BuOH-HOAc-H_2O=4:1:5; V/V; upper layer), 1st direction, and HOAc (=15 % aqueous acetic acid), 2nd direction, with rutin (= quercetin 3-*O*-rutinoside) as a standard. After development, the chromatograms were viewed in long-wave UV light (366 H_2O) and any dark absorbing and fluorescent spots were marked. R_f-values in BAW and 15 % HOAc were calculated.

Methods of identification of flavonoids

After obtaining sufficient amounts of purified flavonoids, they were identified by means of UV spectroscopy using shift reagents to investigate the substitution patterns of the flavonoids (Mabry & al. 1970; Markham 1982) and by acid hydrolysis to identify the aglycone and sugar moieties. Co-chromatography with standards was also performed, where possible. Flavonoid standards available for comparison during the study were apigenin, chrysin, genistein, hesperidin, isorhamnetin, kaempferol, luteolin, morine, myricetin, naringenin, quercetin, rhamnetin, rutin, tricine, and vitexin (all obtained commercially, rutin from Merck, apigenin and luteolin from Sigma and the rest from Fluka).

Acid hydrolysis and identification of flavonoid aglycones

A small amount of each purified flavonoid (*ca* 0.5 mg) was dissolved in 0.5 ml of 80 % MeOH in a test tube. To this sample, 2 ml of 2 M HCl were added and the mixture was heated in a water bath at 100 °C for 0.5 h. The solution was cooled and 2 ml of EtOAc were added and thoroughly mixed with the aqueous layer using a whirley mixer. The upper EtOAc layer was removed with a pipette, evaporated to dryness, dissolved in 0.5 ml of MeOH and applied as spots on thin-layer chromatograms (cellulose). The TLC plates were run in three solvents alongside standards to identify the aglycone moiety (Harborne 1998).

Results

Results have shown that all studied *Trifolium* samples contain flavonoid compounds in their pollen. Data in Tables 1 and 2 show information and also 2-dimentional paper and thin-layer chromatographical data of five samples. Fig. 1 shows a stacked column with a 3-D visual effect histogram for comparing pollen flavonoid data (number of total flavonoids, flavonoid sulphates number, flavone *C*- and *C*-/*O*-glucosides number, and occurrence of chrysin, isorhamnetin, morin, naringenin, quercetin, rhamnetin and rutin in the taxa).

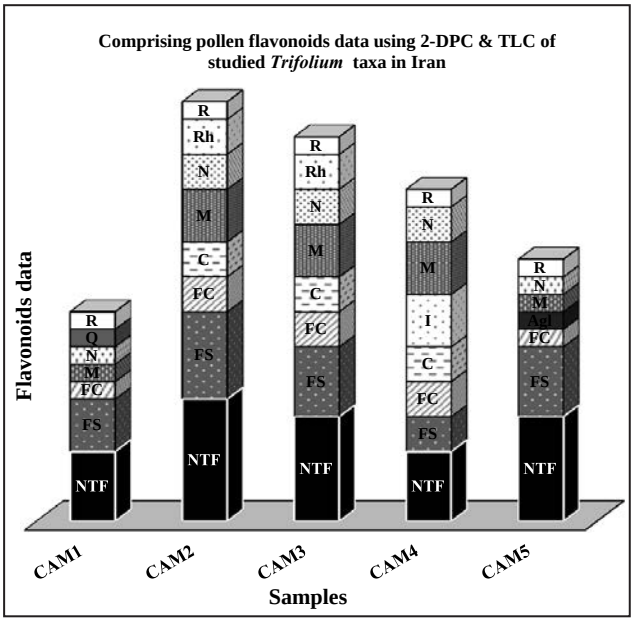

Fig. 1. Stacked column with a 3-D visual effect histogram for comparing pollen flavonoids data of *Trifolium* taxa from Iran using 2-dimensional paper and thin layer chromatographically methods. Scored characters for drawing 3-D column histogram in Excel based on Table 2 data: **–0** (none flavonoid), **+1** (few concentration of flavonoid), **++2** (middle concentration of flavonoid), **+++3** (high concentration of flavonoid). **Abbreviations: NTF** = number of total flavonoids, **FS** = Flavonoid sulphate number, **FC** = Flavone C and *C*-/*O*-glucosides number, **Agl** = Aglycones number, **C** = Chrysin, **I** = Isorhamnetin, **M** = Morin, **N** = Narengenin, **Q** = Quercetin, **Rh** = Rhamnetin, **R** = Rutin. ***CAM**: Mehrangiz Akbari Legumes Collection Number.

As Table 1 and 2 and also Fig. 1 show, apeginin, kaempferol, luteolin, myricetin, tricin, and vitexin were absent in any of the taxa. All studied taxa had flavonoid sulphates, flavone *C*- and *C*-/*O*-glucosides and rutin in their pollen, while aglycones were found only in *T. tumens*. *T. repens* (CAM_2) had the highest number of flavonoids and the smallest number of flavonoids was observed in the pollen of *T. fragiferum*. Isorhamnetin was absent in all pollen taxa, with the exception of *T. repens* var. *repens* (CAM_4). Morin was found in the pollen of all studied taxa, whereas *T. tumens* lacked this flavonoid. The pollen of *T. repens* species and its variety had chrysin and narengenin, which were lacking in the two other samples. Also, quercetin was absent in all studied pollen, with the exception of *T. fragiferum* species. Two *T. repens* populations had rhamnetin in their pollen grains, while the other samples lacked it (Table 1 and Fig. 1).

Table 2. Identification of pollen flavonoids of studied *Trifolium* taxa from Iran by the Thin-Layer Cromatography method.

Voucher data	Flavonoid identification						
	Chrysin	Iso rhamnetin	Morin	Narengenin	Quercetin	Rhamnetin	Rutin
*CAM_1	–	–	+	–	+	–	+
CAM_2	++	–	+++	++	–	++	+
CAM_3	++	–	+++	++	–	++	+
CAM_4	++	+++	+++	++	–	–	+
CAM_5	–	–	–	–	–	–	+

***CAM**: Mehrangiz Akbari Legumes Collection Number, For species names refer to Table 1.
Scored characters: – (non flavonoid), + (few flavonoids), ++ (high concentration of flavonoids) and +++ (very high cocentration of flavonoids).

Discussion

Clover (*Trifolium* sp.) is frequently used as a leguminous cover crop, employed as green manure, and is also included with grasses in various cattle feed mixtures (Sandra & al. 2012). As it is known, the flowers of Iranian *Trifolium* species have intense and beautiful colors and excellent fragrance as attractive agents for insects, and especially honeybees (Tomás-Barberán & al. 2001). Also, the members of *Leguminoseae* are rich in flavonoids and isoflavonoid aglycones (Hegnauer & Grayer-Barkmeijer 1993). The studies of Tomás-Barberán & al. (2001) have shown that pollen contributes to the color, taste and flavor of honey. Also, Pontis & al. 2013 have pointed out some positive correlations between the floral color intensity, flavones, flavonols, and phenolic content (Pontis & al. 2013). Honey is one of the best flavonoid sources (Siess & al., 1996), because of the pollen gathered by the bees to make honey. Tundis & al. (2015) have found that *Trifolium* species are also a rich source of flavonoids. Kolodziejczyk & al. (2011) have pointed out that flavonoids and other phenolic compounds, such as catechins, saponins, clovamides and phenolic acids, are present in clovers. Čeksterytė & al. (2016) reported 6.3 % – 20.2 % of *Trifolium repens* pollen in honey. Bee pollen contains phenolic compounds, vanillic acid, gallic acid, syringic acid, hesperidin, chrysin, narengenin, myricetin, rutin, kaempferol, apegenin, luteolin, quercetin, morin, and isorhamnetin (Freire & al. 2012; Jurikova & al. 2012; Chu & al. 2007; Bonvehi & al. 2001). Our studies have shown that all *Trifolium* samples contained various flavonoid compounds in their pollen. All studied taxa had flavonoid sulphates, flavone *C*- and *C*-/*O*-glucosides and rutin in their pollen grains, while aglycones were found only in *T. tumens*. As Table 1 and Fig. 1 show, *T. repens* has the highest numbers of flavonoids. Rutin, rhamnetin, narengenin, morin, and chrysin were found in the species pollen. Chrysin and nariengenin were found in the pollen of *T. repens* varieties. Also, both *T. repens* populations had rhamnetin in their pollen grains, while isorhamnetin was found in *T. repens* var. *repens* (Table 2). Widyarini & al. (2001) have found that *Trifolium* species are a source of isoflavones, and Tundis & al. (2015) reported quercetin and rutin in *T. repens*. Čeksterytė & al. (2016) detected rhamnetin and isorhamnetin in the mixed pollen-honey samples. Čeksterytė& al. (2006) have found *Trifolium* species pollen grains with quercetin in summer honey. Kaurinovic & al. (2012) have proved the presence of quercetin and naringenin in the *Trifolim* species. Lachman & al. (2010) have assessed the chrysin content in several types of honeys. Table 2 shows morin reported for the first time in the pollen of all studied taxa, whereas *T. tumens* lacked this flavoinoid. Apeginin, kaempferol, luteolin, myricetin, tricin, and vitexin were absent in any pollen of the taxa (Table 2, Fig. 1).

Tomás-Barberán & al. (2001) used the flavonoids as biomarkers to fingerprint the floral origin of honey and reported flavonoids in monofloral honey. As it is presently known, free radical production in animal cells can either be accidental or deliberate. With the increasing acceptance of free radicals as common and important biochemical intermediates, they have been involved in a large number of human diseases (Wegener & Fintelmann 1999). Flavonoids have antioxidant properties and radical scavenging role in cells, which makes honey curative in some cases. Flavonoids are a group of natural compounds, which protect against free radicals (Salvamani & al. 2014). Biological composition of pollen varies from one species to another. Antioxidant properties of honey depend on its botanical origin (Azza & al. 2014). Different studies show that polyphenols present in bee pollen (flavonoids, phenolic acids) largely determine its antioxidant effect (Gheldof & al. 2002; Berreta & al. 2005; Pietta 2000). Flavonoids engage in a wide range of biological activities, have medicinal and pharmacological effect and can protect humans against certain illnesses, because of their antibacterial, antifungal, antiviral,

and anticancer properties (Parr & Bolwell 2000; Noori 2012).

Čeksteryte & al. (2016) have studied the antioxidant potential and composition of phenolic compounds in pure bee products (bee bread and bee pollen). Their results showed that the oxygen radical absorbance capacity of bee pollen methanolic extract is higher than of the bee bread methanolic extract. Also, three glycoside forms (quercetin 3-*O*-sophoroside, quercetin dihexoside and isorhamnetin 3-glucoside) were identified in bee pollen in addition to rhamnetin and isorhamnetin. Kolodziejczyk & al. (2011) believed that antioxidative activity of some *Trifolium* species may also result from the abundance of flavonoids. Kaurinovic & al. (2012) studies have found that the antioxidant activity of all phenolic and flavonoid extracts of *T. pretense* leaves are efficient in the protection of tissues and cells from oxidative stress. Igarashi & Ohmuma (1995) studied the effects of isorhamnetin, rhamnetin and quercetin on the concentrations of cholesterol and lipoperoxide in the serum and liver, and on the blood and liver antioxidative enzyme activities of rats. Their results revealed a decrease in total liver cholesterol concentration and TBARS (thiobarbituric acid-reactive substances) in the cholesterol-free diet of rats by feeding them isorhamnetin, rhamnetin and quercetin. Tundis & al. (2015) have observed anti-lipase activity of flavonoids. They found that flavonoids are able to inhibit some key enzymes involved in carbohydrate digestion such as α-amylase and α-glucosidase.

Conclusion

On the basis of the above-stated data, pollen flavonoid studies can provide useful information for the evaluation of nutritional and medicinal quality of natural honeys and for identifying honey adulteration. Also, pollen flavonoids can be used as key and marker compounds to fingerprint the floral origin of honey. Therefore, an in-depth study of pollen flavonoids in legumes and their nutritional and medicinal properties may lay down the foundation for their further development and utilization.

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