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Research Article

Polyphenolic characterization of pollen grains of some medicinal plant species using Ultra High Performance Liquid Chromatography (UHPLC)

Rajwant Kaur
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar - 143005 (Punjab), India
Manpreet Kaur
Department of Human Genetics, Guru Nanak Dev University, Amritsar-143005 (Punjab), India
Avinash Kaur Nagpal
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar - 143005 (Punjab), India
Jatinder Kaur Katnoria*
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar - 143005 (Punjab), India

*Corresponding author. Email: jkat08@yahoo.com

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Abstract
Polyphenols, the secondary metabolites distributed in different parts of a plant, have major role in protecting the plants from deleterious effects of ultraviolet radiations and various diseases caused by pathogens. Considering the fact that these metabolites possess tremendous medicinal properties, extensive research has been carried out during the past few decades to explore their potential health benefits. Further, polyphenols are documented to possess different activities such as antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial and antiulcer. The present study pertained to analyze different polyphenolic compounds in pollen grains of 9 medicinally important plant species *viz.*, *Bauhinia variegata, B.purpurea, Cassia biflora, C. fistula, C. glauca, C. saimea* and *Delonix regia* belonging to Fabaceae family, *Hibiscus rosa-sinensis* belonging to Malvaceae family and *Melia azadirach* belonging toMeliaceaefamily using Ultra high-performance liquid chromatography (UHPLC). Various polyphenolic compounds likecaffeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, epicatechin, gallic acid, kaempferol, quercetin, rutin, *tert*-butyl hydroquinone andumbelliferonewere found to be present in the pollen grains of these plant species. The order of plant species in terms of maximum number of polyphenolic compounds was observed as *Cassia saimea* (10) > *B. purpurea* (9) = *C. fistula* (9) = *Hibiscus rosa-sinensis* (9) > *Delonix regia* (8) > *B. variegata* (6) > *C. glauca* (4) = *Melia Azadirach* (4) > *C. biflora* (3). The plants such as *C. saimea, B. purpurea*, *C. fistula* and *H. rosa-sinensis* with different polyphenolic compounds indicated their potential forthe treatment of ailments.

Keywords: Catechin, Fabaceae, Phytoconstituents, Pollen grains, Polyphenols

INTRODUCTION

Oxidative modification of the biomolecules such as carbohydrates, deoxyribonucleic acid (DNA), lipids, proteins and ribonucleic acid (RNA) by reactive oxygen species (ROS) results in the occurrence of a wide range of diseases, including cancer. Plants possess secondary metabolites such as polyphenols, alkaloids, saponins and terpenoids that play an important role in scavenging the reactive oxygen species, thus providing protection against various diseases (Govindarajan *et al.,* 2005; Kaur et al., 2005; Valko *et al.,* 2007; Zahin *et al.,* 2014). The exploration of different compounds from the whole plant or any part of the plant possessing antioxidant, antimutagenic and anticancer properties is now a new area of research. Among different parts of plants, exploration of pollen grains for different bioactivities has gained attention due to their several therapeutic properties. Pollen grains, the male gametophytes of flowers, contain various phytoconstituents, including carbohydrates, amino acids, lipids, vitamins, minerals and polyphenols. These phytoconstituents, apart from contributing towards the nutrition value of the pollen grains possess medicinal properties to cure various ailments *viz.,*cold, flu, ulcers, premature ageing, anemia, allergic reactions, enteritis, chronic prostatitis and colitis (Carpes *et al.,* 2007; Saric *et al.,* 2009; Graikou *et al.,* 2011; Kao *et al.,* 2011; Barbieri *et al.,* 2020; Puscion *et al.,* 2020; Thakur and Nanda, 2021).

Among different phyto-constituents, polyphenols are widely distributed in different parts of plants thatcontribute towards various therapeutic properties such as antineoplastic, antidiarrhoeic andantioxidant (Bonvehi *et al.,* 2001; Almaraz-Abarca *et al.,* 2007; Freire *et al.,* 2012). A number of studies have shown the presence of polyphenols in pollen grains of different plant species (Carpes *et al.,* 2007; Almaraz-Abarca *et al.,* 2007; Freire *et al.,* 2012; Leja *et al.,* 2007; Basuny *et al.,* 2013). Bonvehi *et al*. (2001) analyzed 11 honey bee samples with pollens collected from Western Spain for contents of polyphenols, flavonoids and free flavonoid aglycon compounds. During the study, 15 flavonoids were reported to range from 0.35 - 0.76 g/100 g, while total free aglycons ranged from 45.73 - 82.41 mg/100 g. Almeida-Muradian *et al*. (2005) explored dried bee pollen pellets collected from South region of Brazil forthe contents of moisture, ash, lipids, proteins, total carotenoids, beta-carotene and vitamin C. Average contents of protein, moisture, lipids and ash were found to be 20 %, 7.4 %, 6 % and 2.2 %, respectively. The carotenoid content was found to be 76.33 µg/g of the sample, while vitamin C and β-carotene were absent in samples. Pachthong *et al*. (2006) reported two bioactive brassinosteroids from the pollen grains of pumpkin (*Cucurbita moschata*) collected from Mae-Ai district, Chiang Mai, Thailand using gas chromatography - mass spectroscopy (GC-MS).

Leja *et al*. (2007) studied different phenolic constituents of bee pollen containing pollen of 12 plant species *viz., Sinapis alba, Phacelia tanacetifolia, Robinia pseudoacacia, Aesculus hippocastanum, Taraxacum officinale, Malus domestica, Pyrus communis, Trifolium sp., Lamium purpureum, Lupinu spolyphyllus, Chamerion angustifolium* and *Zea mays* collected from Krakow, Poland. The samples were analyzed for different phenolic constituents and the study revealed the presence of total phenols, phenylpropanoids, flavonols and anthocyanins. Kao *et al*. (2011) collected pollen of tea (*Camellia sinensis*) from Western regions of Taiwan and analyzed for content of total polyphenols. Different pollen extracts were prepared using 50 % ethanol, 95 % ethanol, hot water and cold water. The study revealed that ethanolic extracts possessed more phenolic compounds as compared to aqueous extracts. Cosmulescu *et al*. (2015) determined the total phenolic, flavonoids and mineral contents and antioxidant activities of methanolic extracts of pollen of 3 walnut genotypes collected from South Western Romania.

The research, in recent years, is mainly focused on the anticarcinogenic, anti-inflammatory, antibacterial, antioxidant, antiallergic, antiviral, estrogenic and antiulcer properties of these compounds (Kao *et al.,* 2011; Bonvehi *et al.,* 2001; Kroyer and Hegedus, 2001; Munoz *et al.,*2020). Considering the same, the pollen extracts of 9 plant species *viz*., *Bauhinia variegata, B. purpurea, Cassia biflora, C. fistula, C. glauca, C. saimea, Delonix regia, Hibiscus rosa-sinensis* and *Melia azadirach* were screened for the presence of different polyphenolic compounds and their concentration. Different plant parts *viz*., leaves, bark, root, flowers and stamens of these plants are well documented to contain many active phytoconstituents (Balamurugun and Muralidharan, 2010; Kumar and Chandrshekar, 2011; Veerachari and Bopaiah, 2012; Majji *et al.,* 2013; Dahiru *et al.,* 2013; Mariarjancyrani *et al.,* 2013; Bansal *et al.,*2014; Thakur *et al.,* 2016), so far, no report is available on the chemical composition of pollen grains of these plant species under study except for amino acids (Kaur *et al.,* 2015).

MATERIALS AND METHODS

Plant species

The pollen grains of 9 plant species *viz*., *Bauhinia variegata, B. purpurea, Cassia biflora, C. fistula, C. glauca, C. saimea, Delonix regia, Hibiscus rosa-sinensis and Melia azadirach* were collected from the Guru Nanak Dev University campus and were analyzed for the presence of different polyphenolic compounds.

Collection of pollen grains samples

Fresh flowers (just prior to anthesis) of all plant species were collected from Guru Nanak Dev University campus, Amritsar, Punjab (India). For the collection of pollen grains, anthers from each species were removed from the flowers, teased with the help of sharp forceps and tapped in a pre-weighed butter paper to collect pollen grains. The anthers of each species tapped in individual butter paper using 100-150 flowers (per species) to obtain approximately 1 g of pollen grains.The pollen grains thus obtainedwere further used for the preparation of extracts.

Preparation of pollen extracts

Ethanolic extracts of pollen grains of different plant species were prepared following the protocol of Carpes *et al*. (2007) with few modifications. 7.5 ml of 70 % ethanol was added to 1 g of pollen grains and kept at 70 ºC for 1 h with shaking for 1 min after every 10 min interval. The supernatant was pipetted out and the solid residue was re-extracted with 7.5 ml of 70 % ethanol. The process was repeated 3 times and the supernatants were pooled and the final volume made to 20 ml.

Analysis of polyphenols

The extracts were run through Ultra high-performance liquid chromatography (UHPLC) and different polyphenolic compounds were identified and quantified (Model: Nexera, Make: Shimadzu). The separation of different polyphenolic compounds were carried out using C18 column (150 × 4.6 mm, 5 μm particle size) at 25 °C. The flow rate was set at 1.8 ml/min and injection volume used was 50 μl.

Statistical analysis

Pearson correlation was used to find out the correlation between polyphenols of various plant species. The software used for correlation analysis was Statistical Package for the Social Sciences (SPSS-2015).

RESULTS AND DISCUSSION

Pollen grains possess several active constituents such as polyphenols, terpenoids, carotenoids and steroids. The use of apicultural products to cure many diseases or natural diet supplements has gained lots of interest in past decades. Many investigations were observed to be focused on the exploration of active constituents of pollen grains during the study (Bonvehi *et al.,* 2001; Almeida-Muradiana *et al.,* 2005; Almaraz-Abarca *et al.,* 2007; Balamurugun and Muralidhran, 2010; Kao *et al.,* 2011; Barbieri *et al.,* 2020; Puscion *et al.,* 2020; Thakur and Nanda, 2021). The antioxidant related activity of different polyphenolic compounds in pollen grains is also well documented (Carpes *et al.,* 2007; Kao *et al.,* 2011; Leja *et al.,* 2007; Lee *et al.,* 2009). Different polyphenolic compounds *viz*., caffeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, epicatechin, gallic acid, kaempferol, quercetin, rutin, *tert*-butyl hydroquinone and umbelliferone were observed to be present in the pollen grains of plants during present study. However, only catechin was found to be present in all species.

Different plant species under the present study quantitatively contained polyphenols in their pollen grains as 10 (*Cassia saimea*: catechin, epicatechin, caffeic acid, chlorogenic acid, coumaric acid, umbelliferone, ellagic acid, gallic acid, tert-butyl hydroquinone); 9 (*Bauhinia purpurea*: catechin, epicatechin, umbelliferone, caffeic acid, chlorogenic acid, coumaric acid, ellagic acid, gallic acid, *tert*-butyl hydroquinone); 9 (*C. fistula*; catechin, epicatechin, kaempferol, quercetin, rutin, umbelliferone, coumaric acid, ellagic acid, *tert*-butyl hydroquinone); > 9 (*Hibiscus rosasinensis;* catechin, kaempferol, quercetin, umbelliferone, caffeic acid, chlorogenic acid, coumaric acid, gallic acid, *tert*-butyl hydroquinone); > 8 *Delonixregia; catechin, kaempherol, rutin,* umbelliferone, chlorogenic acid, coumaric acid, gallic acid, ellagic acid); > 6 (*Bauhinia variegata;* catechin, epicatechin, umbelliferone,coumaric acid, ellagic acid, *tert*-butyl hydroquinone); > 4 (*C. glauca*; catechin, epicatechin, umbelliferone coumaric acid, gallic acid, *tert*-butyl hydroquinone); > 4 (*Melia azadirach*; catechin, kaempherol*,* quercetin, gallic acid); > 3 (*C. biflora;* catechin, chlorogenic acid, gallic acid) as shown in Table 1. Various environmental factors like sun exposure, soil type, rainfall, plant nutrition etc., affect the polyphenol content in plants(Pandey and Rizvi, 2009).

Polyphenolic compounds are mainly categorized into two groups *viz*., flavonoids and phenolic acid. Flavonoids contain two or more aromatic rings and are further subdivided into flavones, isoflavones, isoflavanes, flavanones, flavonols, anthocyanidins, chalcones and dihydrochalcones, while phenolic acid contains a phenolic ring and an organic carboxylic acid and are further subdivided into hydrobenzoic acid and hydrocinnamic acid (Kao *et al.,* 2011; Almeraz-Abarca *et al.,* 2007). Almaraz-Abarca *et al*. (2007) reported that flavonol and phenolic acids were the main classes of polyphenols present in the pollen grains of *Prosopis juliflora.* Seven phenolic acid *viz*., caffeic acid, chlorogenic acid, coumaric acid, ellagic acid, gallic acid,tert-butyl hydroquinone and umbelliferone were analyzed during the study. Among 9 plant species studied, caffeic acid was present only in three plant samples viz., *Bauhinia purpurea, Cassia saimea* and *Hibiscus rosa-sinensis* gallic acid were present in all the sample except *Bauhinia variegata* and *Cassia fistula*. Different flavonols *viz*., kaempferol, quercetin and rutin were analyzed in the pollen grains of different plant species.

Many reports are available for different bioactivities of the polyphenolic compounds. Zahin *et al*. (2014) reported two polyphenolic compounds *viz*., penicalagin and ellagic acid, extracted from fruit peel of *Punica granatum*, showed antimutagenicity and resulted in inhibition of DNA adducts formation induced by benzo (a)pyrene. Smerak *et al*. (2002) evaluated the antimutagenic activity of ellagic acid following Ames assay. Gallic acid was reported for its anti-neoplastic activity and antifungal activity of leaf extract of *Toona sinensis* (Chia *et al.,* 2010; Hong *et al.,* 2011), while rutin, extracted from Tobacco leaves, was reported for its antifungal, antibacterial, anthelmintic, larvicidal and cytotoxicity (Dubey *et al.,* 2014). Geetha *et al*. (2004) reported the quercetin had antioxidant and antimutagenic potential. The auto-oxidation of quercetin resulted in the production of hydroxyl radicals within the cells responsible for antiproliferative and antimutagenicity. Rho *et al*. (2011) reported the depigmenting and anti-inflammatory response of Kaempferol isolated from *Hibiscus cannabinus.*

Pearson correlation showed the presence of a positive correlation between gallic acid and rutin at 5 % level of significance (Table 2). Rutin is known as an important phyto-antioxidant and plays an important role in improving blood circulation, so it is used to treat haemorrhoids, varicosis and microangiopathy (Ugusman*et al.,* 2014), while gallic acid is well known to inhibit amyloid

Table 1. Contents (mg/l) of different polyphenolic compounds in pollen grains of different plant species.

	Flavonoids					Phenolic acid						
	Flavanols		Flavonols			Hydrocinnamic acid				Hydrobenzoic acid		
Sample codes	**Cate-chin**	**Epicate-chin**	**Kaempferol**	**Quercetin**	**Rutin**	**Caf-feic acid**	**Chlorogen-ic acid**	**Coumaric acid**	**Umbellifer-one**	**Ellagic acid**	**Gallic acid**	**Tert-Butyl Hy-droquinone**
BP	15.147	10.987	-	-	-	0.115	0.168	0.918	4.308	24.626	0.217	46.198
BV	16.370	2.386	-	-	-	-	-	5.251	13.2	11.236	-	109.839
CB	4.749	-	-	-	-	-	0.249	-	-	-	0.291	-
CF	266.158	0.965	41.664	25.264	4.984	-	-	0.499	16.366	22.192	-	0.051
CG	3.713	0.410	-	-	-	-	-	-	4.092	-	0.347	-
CS	28.462	0.118	1.511	1.652	27.646	41.461	0.486	-	10.863	23.980	2.022	-
DR	20.816	-	242.864	-	24.865	-	5.004	0.262	5.484	1565.990	0.676	-
HR	15.379	-	228.600	9.679	-	0350	4.111	0.168	2.733	-	0.103	6.990
MA	5.883	-	237.656	14.909	-	-	-	-	-	-	0.709	-

BP: *Bauhinia purpurea,* BV: *B. variegata,* CB: *Cassia biflora,* CF: *C. fistula,* CG: *C. glauca,* CS: *C. siamea,* DR: *Delonix regia,* HR: *Hibiscus rosa-sinensis* and MA: *Melia azadirach*

Table 2. Correlation matrix showing a correlation between different polyphenolic compounds.

Parameter	CT	EC	KM	QU	RU	UM	CC	CH	CA	EA	GA	TBH
CT	1											
EC	-0.063	1										
KM	-0.133	-0.362	1									
QU	0.788*	-0.231	0.298	1								
RU	0.029	-0.265	0.140	-0.189	1							
UM	0.693	0.020	-0.393	0.310	0.289	1						
CC	-0.125	-0.192	0.442	0.144	-0.128	-0.199	1					
CH	-0.153	-0.258	0.723*	-0.116	0.410	-0.204	0.560	1				
CA	-0.048	0.232	-0.299	-0.213	-0.234	0.483	-0.158	-0.213	1			
EA	-0.083	-0.161	0.508	-0.233	0.616	-0.0405	-0.147	0.734*	-0.112	1		
GA	-0.232	-0.245	-0.009	-0.219	0.777*	0.021	-0.119	0.003	-0.354	0.119	1	
TBH	-0.165	0.463	-0.346	-0.316	-0.303	0.362	-0.133	-0.244	0.961*	-0.176	-0.359	1

CC: Caffeic acid; CT: Catechin; CH: Chlorogenic acid; CA:Coumaric acid; EA: Ellagic Acid: EC: Epicatechin; GA: Gallic acid; KM: Kaempferol; QU: Quercetin; RU: Rutin; TBH: Tert-Butyl Hydroquinone; UM: Umbelliferone; *Data is significant at P ≤ 0 fibrils. A positive correlation existed between quercetin and catechin (as shown in Table 2). Pignatelli *et al*. (2000) reported that both compounds have a tendency to inhibit collagen-induced hydrogen peroxide production and calcium mobilization and 1,3,4-inositol triphosphate formation in combination. In the present study, positive correlation is existed between kaempferol and chlorogenic acid at 5 % level of significance. Chlorogenic acid has shown anti-oxidative and anti-hypertensive effects (Zhao *et al.,* 2011) while kaempferol has been documented to have anticancer, antidiabetic, antiviral and antioxidant effects. Ellagic acid also showed a significant correlation with rutin. The present study showed that coumaric acid showed a strong positive correlation with *Tert*-Butyl Hydroquinone. Both coumaric acid and *Tert*-Butyl Hydroquinone have antioxidant effects. Polyphenol umbelliferon were documented to be used as a sun screen agent due to its ultraviolet radiation protective activity (Lou *et al.,* 2010). Different polyphenols are consumed as dietary supplements or obtained from foodstuffs such as fruit and vegetables due to their properties to reduce incidences of heart disease, cancer, gastrointestinal and neurological diseases, liver diseases, atherosclerosis, obesity and allergies.

Conclusion

Polyphenols are considered to be potent antioxidants that protect the different biomolecules such as DNA, RNA, proteins, lipids and carbohydrates from oxidative stress. In the present study, various polyphenolic compounds *viz*., caffeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, epicatechin, gallic acid, kaempferol, quercetin, rutin, *tert*-butyl hydroquinone and umbelliferonewere found to be present in pollen grains of 9 plant species. As all polyphenols are well documented for their bioactivities, the present study would be very useful in the field of pharmaceuticals.

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Conflict of interest

The authors declare that they have no conflict of interest.

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