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Characterization of chemical composition of ethanolic extract of bee pollen in India

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Abstract

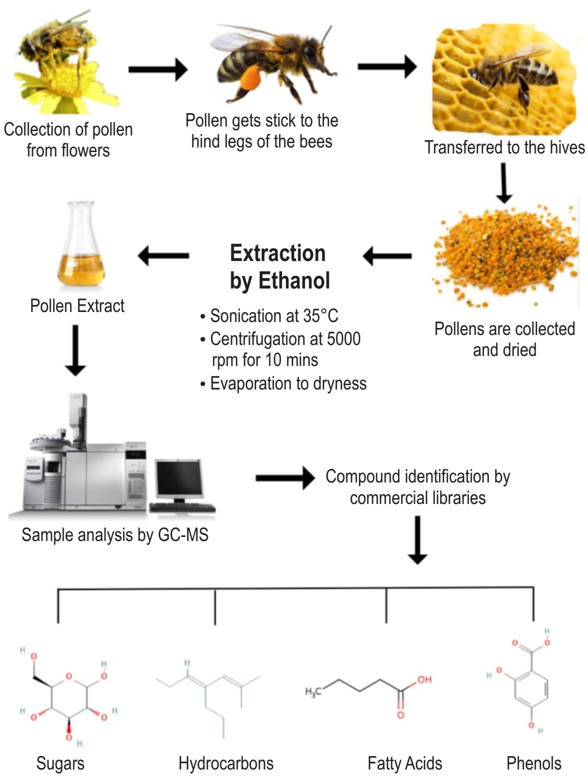
Aim: Bee pollen is a mixture of pollen/male gametophyte of flowers, nectar/sweet liquid substance from flowers and salivary secretions of bees. It comprises a huge diversity of compounds which are thought to work together for medicinal purpose. The present study was undertaken to assess the bioactive components of bee pollens responsible for therapeutic properties.

Methodology: The volatile chemicals compounds found in ethanolic extracts of *Apis mellifera* pollen were analysed by gas chromatography-mass spectrometry.

Results: The observations revealed the presence of hydrocarbons, sugars and their derivatives, fatty acids, glycosides, alcohol, esters, aldehyde and carbamate.

Interpretation: Present findings authenticate huge chemical diversity and hence, further study on pollens is required due to its broad spectrum of potential medicinal and nutritional activities.

Key words: G-C mass spectrometry, Bee pollen, Bioactive compounds



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Introduction

Studies on natural products such as; pollen, propolis and honey has received attention due to increased demand for balanced, healthy nutritious diet and also for their therapeutic potentialities (Rana *et al.,* 2022 a,b; Rana and Kumar, 2022). The chemical composition of these products are greatly influenced by geographic vegetation from which they are derived, climatic conditions, soil type, bee races as well as time of collection (da Silva *et al.,* 2014; Nogueira *et al.,* 2012; Prdun *et al.,* 2021; Rana, 2021). Among them, honeydew/nectar and pollen/bee bread are two main components required for the brood development as well as for the survival and maintenance of health of bee colonies (Dolezal and Toth, 2018; Tutun *et al.,* 2022). Pollens are a diverse plant product rich in biologically active compounds and are characterized for being a natural source for various proteins, amino acids, carbohydrates, lipids and fatty acids, phenolic compounds, vitamins, enzymes and coenzymes (Vassev *et al.,* 2015; Ares *et al.,* 2022). So for research or for its consumption, it is collected by using a pollen trap (Karabagias *et al.,* 2021), which after getting mixed with salivary secretions and nectar from flowers and after undergoing fermentation at suitable temperature gets converted into bee bread also called stored pollen/ambrosia (Rana and Parmar, 2022; Bakour *et al.,* 2022) and can be directly consumed by developing brood.

It has become one of the most popular food supplements in recent years due to the associated health advantages, which include antioxidant, anti-inflammatory, anticancer, analgesic and antimicrobial properties (Ares *et al.,* 2018, Gonçalves *et al.,* 2021; Rana, 2021; Rana and Kumar 2023; Rana and Bajwa, 2023). During the foraging trips, honeybees in addition to water also gathers two important food sources required for the growth of their colonies growth; one is nectar which, after getting converted into honey by their salivary enzymes (Invertase and diastase) becomes an important source of carbohydrates and the other is pollen which is an important source of proteins, lipids, minerals and fats (Hoover & Ovinge, 2018). Pollen is not only an important source for the production of brood food but also required for newly emerged workers to develop tissues required for brood rearing and overwintering, *i.e.,* fat bodies and hypo-pharyngeal glands (Keller, 2005).

Pollen collection and its composition are dependent on various factors such as; bee species, age and the behavioral tasks performed by workers of different bee species. The nurse bees are actively engaged in brood rearing and feeding, while the queen consumes relatively larger amount of pollens. The difference in foraging activity among different bee species such as; the little honey bee- *Apis florea*, the India/Asian honey bee- *Apis cerana indica*, the rock honeybee- *Apis dorsata*, the European or Italian honeybee – *Apis mellifera*, stingless bee- *Melipona irridipennis* and the bumblebees all do not appear same with regards to their foraging behavior (Hanley *et al.,* 2008). Bumblebees are reported to forage pollens from twice the number of plant species as compared to honeybees. They forage at least six to seven different species during one foraging visit, while honeybees show a constant flower foraging behavior (Leonhardt and Blüthgen, 2012). It has also been reported that honeybees do not forage preferentially on pollens with higher protein concentration, while bumblebees preference on pollens with higher concentration as well as quality of protein (Ghosh *et al.,* 2020). In this study, bee pollens collected from the bee specie *Apis mellifera* was used for the study. *Apis mellifera* is a European import bee specie which provides 75% of India's honey and is widely used and popular among commercial beekeepers due to its higher honey production capacity (Shahi *et al.*, 2011; Thomas *et al.,* 2002). The reason behind using this bee species for pollen collection is very simple, as it outperformed other indigenous species found in India in terms of honey production, pollen load, capacity of laying eggs, sustainability under adverse conditions, *i.e.,* dearth period and also its ability to recover after deteriorating environmental conditions (Dalio, 2015).

Gas chromatography-mass spectrometry (GC-MS) analyses of ethanolic bee pollen extracts are limited and therefore, the aim of the present study was to determine the volatile components of bee pollen extract in order to better understand the potential resources required for future therapeutic uses.

Materials and Methods

Collection of bee pollens: Pollens were collected from pollen basket of worker honey bees (*Apis mellifera*) returning to the hive with pollen loads, by installing five/six pollen traps at the entrance of the beehive at an Apiary located in Jhajjar district of Haryana, India. The analysis was during May-June, 2023.

Preparation of bee pollen extract: The extraction process was carried out according to Omar *et al.* (2018) with minor modifications. Ten grams of pollen sample was suspended in 100 ml of ethanol in 1:10 (w:v) ratio. This material was then mixed and sonicated for 90 min in an ultrasonic bath at 35°C. The material was then centrifuged for 10 min at 5000 rpm. The resulting supernatant was filtered in a round bottom flask before being evaporated to dryness on a rotatory evaporator. The pollen extract was then used for chemical analysis.

GC-MS analysis: The chemical profiling of the pollen sample was analyzed by GC-MS. Agilent Technologies, Inc. Helium at a constant flow rate of 1 ml min^{-1}, was used as the carrier gas. The source temperature was maintained at 280°C and the solvent delay time was 1 min. Chromatographic data were processed using the Agilent Mass Hunter software. Identification of the compounds was based on a comparison of mass spectra and computer searches on commercial libraries and literature data. Due to lack of appropriate references and information, certain components detected remained unidentified.

Statistical analyses: The identification of peaks was performed by taking single sample and by comparing the results with commercial reference libraries such as the NIST library.

Results and Discussion

The chromatographic data observed through GC-MS analysis of ethanolic extract of bee pollen led to the identification of 47 different chemical compounds with quality greater than 60% such as; sugars, hydrocarbons, fatty acids, phenolic acids, alcohol, ketones, glycosides, carbamate, *etc.* (Table 1). Chromatographic data analysis revealed that the derivatives of sugar (Glucosyloxyazoxymethane, Xylopyranoside, Ribofuranos) followed by fatty acids (Pentanoic acid, dienoic acid), hydrocarbons (1,2-Bis (methylsilyl) ethane, 1,3-Hexadiene, 4-diethylboryl-3-trimethylsilyl,etc.), boronic compounds (Borinic acid, 9-Borabicyclo [3.3.1] nonane, 9-(3-methoxycyclohexyl) oxy) were the dominant compounds in bee pollen ethanolic extract. Though compounds like phenolic acid, alcohol, carbamate esters, ketones were also detected in small fractions, however, they also contributed to the overall profile of the bee pollen (Table 1, 2). The bulk of the peak area identified in this bee pollen extract was sugar and its derivatives, *i.e.,* 42.19% of the total peak area. The derivative of glucose showed the highest peak area with 20.18%, followed by ribose (11.06%) and xylose (10.95%). Fatty acids, hydrocarbons, boronic compounds followed right behind with a peak area of about 40%. Compounds like alcohol, ketones, phenolic acid, carbamate esters and other minerals covered the remaining area (Fig. 1).

Methylene chloride was also identified in high concentrations in the bee pollen sample. It alone covered a significant portion of the peak area. It is not a natural component of bee pollen and is not expected to be present in significant amount, unless it has been introduced through human activities, such as pesticide use or industrial pollution (Fig.1). Differences in distinct chemical groups discovered in many other phytochemical analyses of bee pollen extract revealed that the chemical compounds contained in these samples are dependent on the geographical location and the floral preferences of specific bee species. Bees collect pollens from a wide variety of flowers, and it has been recorded that pollens collected by one species of bee may belong to more than 30 flower species (Omar *et al.,* 2018; Poolpraset, 2014). The bee pollen sample in this study was collected from a farm with a diversified flora, including *Brassica juncea, Eucalyptus tereticornis, Ocimum sanctum, Trachyspermum ammi, Dalbergia sissoo*, and others. So the difference in results might be due to different flower species (from where the bee pollen sample was collected) which may be the source of a diverse set of volatile chemicals, some of which may be valuable for human therapeutic uses.

Sugar and its derivatives, hydrocarbons, fatty acids, and other substances such as alcohol, ketones, phenolic acids, carbamate and other nutrients found in this study are in agreement with a prior GC-MS analysis of methanolic extract of honeybee pollen (Graikou *et al*., 2011) which also showed the presence of sugars, fatty acids their esters and phenolic substances. Their studies showed highest peak area for glucose, which was found to be one of the most common sugars present in bee pollen (Graikou *et al.,* 2011; Thakur and Nanda, 2020, Qian *et al.,* 2008; da Silva *et al.,* 2014). Glucose in bee pollen serves as a source of energy for the bees (Xu *et al.,* 2020). It is also used as a carbohydrate source for human consumption as it is a simple sugar that can be easily broken down and absorbed for energy (Holesh *et al.,* 2022). The other two sugars found in the sample were xylose and ribose. Xylose is a source of xylitol and is commonly used as a food sweetener (Chen and Wang, 2017). Ribose is vital in our "genetic material" since it is a major component of ATP molecule and has been shown to improve insufficient cellular energy levels (Shecterle *et al.,* 2010).

Pentanoic acid and nona-2,3-dienoic acid were two types of fatty acids found in this bee pollen sample. Pentanoic acid was abundant and utilised to treat neurological disorders (Jayaraj *et al.,* 2020). In a series of *in vitro* and orthotopic xenograft mouse models, pentanoic acid/valeric acid was found to have a broad spectrum of anticancer activity, with particularly high cytotoxicity for liver cancer in cell proliferation, colony formation, wound healing and cell invasion (Han *et al.,* 2020). The only phenolic compound found in bee pollen sample was 2,4-Dihydroxybenzoic acid, which comprised antioxidant, anti-radical and hydrogen peroxide scavenging properties. When activated with ultrasound, it can be used in the wastewater treatment and also utilised as a reagent in the synthesis of mycophenolic acid, protein 90 inhibitors, and pde4 inhibitors (Han *et al.,* 2020).

Micronutrients like phosphorus, boron, iron, etc., were also found in small quantities in the bee pollen samples. Though they do not play a role in energy balance, they are required for all chemical processes and for the existence of life. At all stages of life, the body requires micronutrients in small quantities for growth and development (El Ghouizi *et al.,* 2023). Among other identified compounds, many of these compounds have antioxidant, antimicrobial, anticancer, anti-inflammatory, and anti-allergic activities (Guine, 2015). Phenolic compounds, ketones, aldehydes etc., acquire these properties.

The volatile substances found in bee pollen can come from a variety of sources, including plant nectar, molecules modified or produced by bees, bee-pollen processing, storage, and contamination by the environment or bacteria (Igor Jerković, 2010). Though varieties of bee pollen compounds are influenced by floral and geographical origin (Kaškonienė *et al.,* 2008), volatile isolation and detection procedures may also have an impact (Nurul Syazana *et al.,* 2013). These conditions may produce volatile molecules that differ from those seen in this and other studies. As a result, the volatile molecules identified in this study were diverse, including sugar compounds, hydrocarbons, fatty acids, phenolic acids, alcohol, carbamate and other minerals. Sugar and its compounds, as well as hydrocarbons and fatty acids, were key components of the ethanolic extract of bee pollen.

The analysis chromatographic data revealed that the derivatives of sugar, followed by fatty acids, hydrocarbons and

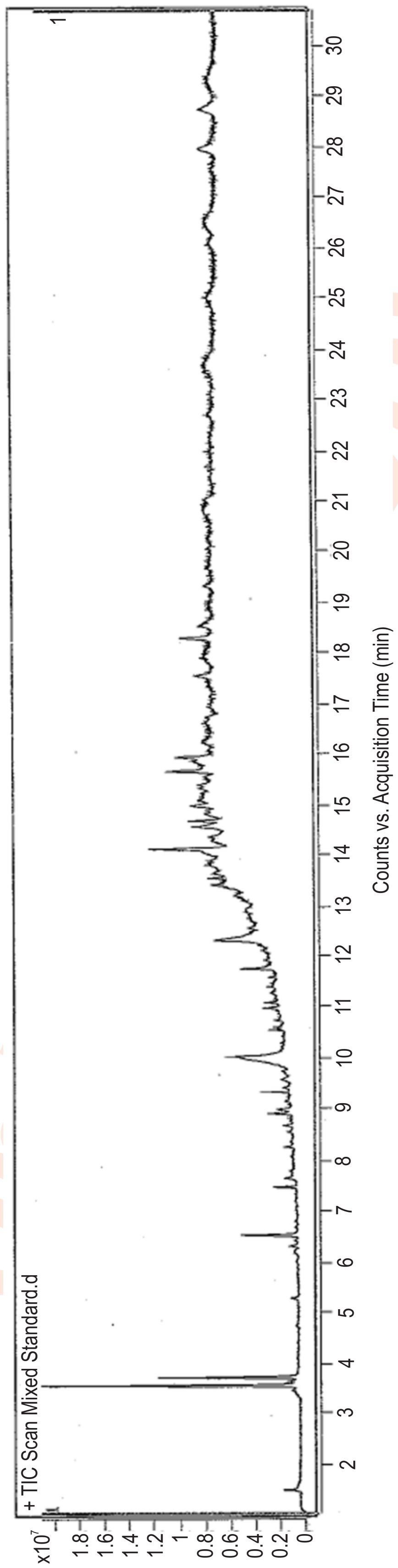

Fig.1: Chromatographic analysis of ethanolic extract of bee pollen.

Fig. 1: Chromatographic analysis of ethanolic extract of bee pollen

Name	Classification	Formulas	RT
beta,-D-Glucosyloxyazoxymethane	Sugar	$C_8H_{16}N_2O_7$	1.188
Hydroxyurea	Organic hydroxylamine	$CH_4N_2O_2$	1.473
1,2- Bis(methylsilyl)ethane	Hydrocarbon	$C_4H_{14}Si_2$	3.452
Methylene Chloride	Chlorinated hydrocarbon	CH_2Cl_2	3.548
1,3,4-Oxadiazole, 2-(acetyloxy)-2,5-dihydro-2,5,5-trimethyl-	Oxadiazole derivative	$C_7H_{12}N_2O_3$	3.635
Dimethyl ether	Ether	C_2H_6O	3.714
1-Tetrazol-2-ylethanone	Tetrazole derivative	$C_3H_4N_4O$	5.294
3-Azonia-5-hexene-1-ol,N,N-dimethyl-,carbamate ester, bromide	Carbamate ester	$C_8H_{17}N_2O_2$	6.208
Silacyclobutane	Organosilicon compound	C_3H_8Si	6.316
2,4-Pentanedione, Ion (1-), lithium	Lithium salt of diketone	$C_5H_7LiO_2$	6.421
1,2-Oxaborolane, 2-ethyl-4,5-dimethyl-	Boron containing compound	$C_7H_{15}BO$	6.534
Ethylphosphonic acid, bis (tert-butyldimethylsilyl) ester	Phosphonic acid ester	$C_{14}H_{35}O_3PSi_2$	7.483
1-Methyl-1- silacyclobutane	Organosilicon compound	$C_4H_{10}Si$	7.655
Cetrimonium Bromide	Ammonium salt	$C_{19}H_{42}BrN$	8.269
Cyclopentyl-methyl- phosphinic acid, 2-Isopropyl-5-methyl-cyclohexyl ester	Phosphinic acid ester	$C_{16}H_{31}O_2P$	8.365
Iron, tricarbonyl[(0,1,2,3- .eta.)-methyl 2-propenoate]	Organoiron complex	$C_7H_6FeO_5$	8.683
Formic acid hydrazide	Hydrazide derivative of Formic Acid	CH_4N_2O	8.918
1,3,2-Dioxaborolane, 2-ethyl-4-(3- oxiranylpropyl)-	Organoboron compound	$C_9H_{17}BO_3$	8.997
3,3-Dimethyl-hepta-4,5-dien-2-ol	Alcohol	$C_9H_{16}O$	9.154
1,3,5,7,9-Pentaethylbicyclo[5.3.1]pentasiloxane	Organosilicon compound	$C_{10}H_{28}O_6Si_5$	9.335
3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	Heterocyclic compound of class thieno[2,3-d]pyrimidines	$C_{14}H_{16}O_2S_2$	9.431
Cyclododecyl isothiocyanate	Isothiocyanate	$C_{13}H_{23}NS$	9.754
Pentanoic acid, 5-hydroxy-,2,4-di-t-butylphenyl esters	Fatty acid	$C_{19}H_{30}O_3$	9.998
4-[5-(4-Fluoro-phenyl)-tetrazol-2-yl]-butyramide	Amide	$C_{11}H_{12}FN_5O$	10.557
2-Azidomethyl-1,3,3-trimethyl-cyclohexene	Heterocyclic compound	$C_{10}H_{17}N_3$	10.607
Nona-2,3-dienoic acid, ethyl ester	Fatty Acid	$C_{11}H_{18}O_2$	10.755
Phosphinic acid, diisopropyl-, menthyl ester	Phosphinic acid ester	$C_{16}H_{33}O_2P$	11.096
Silane,chlorodiethylheptyloxy-	Organosilicon compound	$C_{11}H_{25}ClOSi$	11.407
9- Borabicyclo[3.3.1]nonan e, 9-(3- methoxycyclohexyl)oxy-	Boronic acid ester	$C_{15}H_{27}BO_2$	11.480
Diethyl (1-aminocyclohexyl)phosphonate	Phosphonic acid ester	$C_{10}H_{22}NO_3P$	11.757
8-(2-Acetyloxiran-2-yl)- 6,6-dimethylocta-3,4- dien-2-one	Ketone	$C_{14}H_{20}O_3$	11.922
2,4-Dihydroxybenzoic acid, 3TMS derivative	Phenolic acid	$C_{16}H_{30}O_4Si_3$	12.335
Borinic acid, diethyl-, 1-cyclododecen-1-yl ester	Boronic acid ester	$C_{16}H_{31}BO$	12.915
Cyclohexane, 1R-acetamido-2cis,4cis-bis(acetoxy)-3trans- azido-	Azide	$C_{12}H_{18}N_4O_5$	13.410
2-Troponyl difluoroborate	Boron-containing organic compound	$C_7H_5BF_2O_2$	13.556
Nona-2,3-dienoic acid,ethyl ester	Fatty acid	$C_{11}H_{18}O_2$	14.289
13-Borabicyclo[7.3.0]tridecane, 13-butoxy-, (Z)- or (E)-	Boron-containing organic compound	$C_{16}H_{31}BO$	14.679
2,4-Heptadiene, 5-diethylboryl-2-methyl-4-trimethylsilyl-	Hydrocarbon	$C_{15}H_{31}BSi$	14.982
Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester	Azide	$C_{16}H_{31}O_2P$	15.334
1,2,4,5-Tetrazine-3,6-diamine, 1,4-dioxide	Heterocyclic compound	$C_2H_4N_6O_2$	15.675
.alpha.-d-Xylopyranoside, 2,4-0-(ethylboranediyl)-1-0-methyl-	Sugar	$C_8H_{15}BO_5$	15.943
Undeca-3,4-diene-2,10-dione, 5,6,6-trimethyl-	Hydrocarbon	$C_{14}H_{22}O_2$	18.306
1,3-Hexadiene, 4-diethylboryl-3-trimethylsilyl-	Hydrocarbons	$C_{14}H_{29}BSi$	18.545
Borane, dlethyl[1-[(trimethylsilyl)methylenelpropyll-, (Z)-	Boron compounds	$C_{11}H_{25}BSi$	19.337
Silacycloundec-6-yne,1,1-dimethyl-	Organosilicon compounds	$C_{12}H_{22}Si$	20.251
7-[.beta.-d- Ribofuranosylimidazo[4, 5-d][1,2,3]-triazin-4-one (2-azainosine)	Sugar derivative	$C_9H_{11}N_5O_5$	21.019
1,3-Dioxolo[4,5- e][1,3,2]dioxaborepin, 6- ethyltetrahydro-2,2-dimethyl-, cis-	Boron compound	$C_9H_{17}BO_4$	23.683

boronic compounds were dominant in the ethanolic extract of bee pollen. Though compounds like phenolic acid, alcohol, carbamate esters, ketones were also detected in small portion. Hence GC-MS studies on bee pollen authenticate the huge chemical diversity of bee pollen. However, the huge variation in composition occurs as compared to previously conducted studies and the reasons can be different geographic and botanic origin which can be a challenge in using bee pollen as nutritional

Table 2: Main compounds observed through GCMS analysis in bee pollens

Hydrocarbons	1,2- Bis(methylsilyl)ethane Undeca-3,4-diene-2,10- dione, 5,6,6-trimethyl- 2,4-Heptadiene, 5-diethylboryl-2-methyl-4-trimethylsilyl- 1, 3-Hexadiene, 4-diethylboryl-3-trimethylsilyl
Sugars	beta,-D-Glucosyloxyazoxymethane alpha.-d-Xylopyranoside, 2,4-0-(ethylboranediyl)-1-0-methyl 7-[.beta.-d- Ribofuranosylimidazo[4, 5-d][1,2,3]-triazin-4-one (2-azainosine)
Fatty Acids	Nona-2,3-dienoic acid, ethyl ester Pentanoic acid, 5-hydroxy-,2,4-di-t- butylphenyl esters
Phenolic Acid	2,4-Dihydroxybenzoic acid, 3TMS derivative
Carbamte esters	3-Azonia-5-hexene-1-ol, N,N-dimethyl-,carbamate ester, bromide
Alcohols	3,3-Dimethyl-hepta-4,5-dien-2-ol

supplement as well as in therapeutic applications.

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