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Phytochemical profiling of ethanolic extract of bee pollen using Gas Chromatography Mass Spectroscopy and its *in silico* analysis of hypocholesterolemic activity

K. Ashwathi[a], V.M. Nandhini[1a], V. Thirumalai[2], N. Ezhili and Charumathi Pushparaj*

Department of Zoology, PSGR Krishnammal College for Women, Coimbatore 641004, Tamil Nadu, India.
[1]Department of Zoology, PSG College of Arts and Science, Coimbatore 641004, Tamil Nadu, India.
[2]Department of Pharmacology, Rajas Dental College and Hospital, Kaval Kinaru Junction, Tirunelveli 627105, Tamil Nadu, India.
[a]Authors contributed equally to the research work
Email: charumathi@psgrkcw.ac.in

ABSTRACT: Bee pollen, a bee product, is considered as one of the valuable products in natural medicine, for its excellent therapeutic properties. Cold maceration technique was employed to extract bee pollen and the phytochemical constituents were analysed. The preliminary phytochemical analysis showed the presence of acids, proteins, carbohydrates, phenol, flavonoids, glycosides, anthraquinone, quinone, resins, saponin, tannin, and terpenoids. The advanced phytochemical screening of volatile chemical constituents analyzed using Gas Chromatography and Mass Spectrometry showed 40 volatile chemical compounds from the bee pollen extract. Among those, the hypocholesterolemic effect-related compound of bee pollen was analyzed by in silico docking method, where Oxidosqualene: Ianosterolcyclase (OSC) was taken as a target enzyme which helps in cholesterol biosynthesis. Hexadecanoic acid, Pentadecanoic acid, Pyrroloindole, and Alpha amyrin selected as ligands, and Ro 48-8071 fumarate used as a standard enzyme inhibitor for molecular docking analysis, showed that all the ligands efficiently bound with the target enzyme OSC. Among the four compounds, alpha amyrin had the highest binding interaction energy with the target enzyme (-13.37 kJ mole^{-1}). The OSC enzyme inhibition activity may be responsible for the hypocholesterolemic effect and weight-reducing property of bee pollen.

KEY WORDS: Cold maceration, Ro 48-8071 fumarate, hypocholesterolemic, Oxidosqualene: Ianosterolcyclase, molecular docking analysis, Alpha amyrin

INTRODUCTION

Honey bee pollen contains numerous essential nutrients, including carbohydrates, unrefined fibers, proteins, and lipids. Furthermore, minor constituents, encompassing amino acids, minerals, trace elements, vitamins, carotenoids, phenolic compounds, flavonoids, sterols, and terpenes also constitute bee pollen (Linskens and Jorde, 1997; Kostić *et al.,* 2015; Li *et al*., 2018). The composition of bee pollen is influenced by various factors, including atmospheric conditions, soil

* Author for correspondence

characteristics, and the foraging behaviour of bees (Liolios *et al*., 2019; Mayda *et al*., 2020). Bee pollen is collected using special collecting tray called pollen trap. Pollen from flowers is collected by worker bees and stored as pellets in the pollen baskets in the corbiculae of the legs. When the bees enter into the hive, the pellets of pollen can be scrapped from the legs and collected in the pollen trap (Somerville, 2012). Recent studies have proven the economic importance of bee products due to several bioactive potential, such as antimicrobial, antiviral, antitumor, and anti-inflammatory properties (Alvarez-Suarez, 2017). The components of bee pollen is of great interest to the pharmaceutical industry especially flavonoids and phenolic acids, as they are reportedly used in therapeutics against many diseases related to oxidative damage such as cardiovascular and neurodegenerative disorders (Alimoglu *et al.,* 2021). The present work describes the phytochemical constituents in the cold macerated ethanolic extract of bee pollen, quantified through GCMS. Additionally, the hypocholesterolemic effect of bee pollen was determined using *in silico* molecular docking method (Campos *et al*., 1997; Mãrgãoan *et al*., 2019; Morris and Lim-Wilby, 2008).

MATERIALS AND METHODS

Bee pollen and chemicals: *Apis indica* bee pollen was collected using pollen traps from the Nilgiri Biosphere Nature Park (NBNP), Coimbatore, Tamil Nadu, maintained by the Zoological Parks Association of India at PSGR Krishnammal College for Women. Test reagents and solvents were obtained from Sigma Aldrich and Hi-Media. Cold maceration was selected as the extraction process, much like cold pressing, employs low extraction temperature and retains the odour of the source material without degrading the thermolabile compounds present in the fraction (Wu *et al*., 2015; Sankeshwari *et al*., 2018). GCMS help us to identify different substances within a test sample (Prabhakar Joshi and Dayaram Wagh, 2018). Molecular docking is a technique used to predict the best match between two molecules when they are bound to each other in order to generate a stable complex (Kumar, 2019). This technique, utilized in various domains provides a comprehensive approach to study the molecular systems of small chemical systems up to large biological molecules and material assemblies (Lengauer and Rareyt, 1996).

Extraction of sample: Powdered bee pollen 100g was soaked in ethanol in a stoppered container at room temperature for three days. The mixture was then strained and filtered to obtain a complete extract following the method described by Nurdianah *et al*. (2016). The solvent ethanol was allowed to evaporate, resulting in a semi-solid extract that was stored for further analysis (Li *et al*., 2019).

Preliminary phytochemical analysis: Tests were carried out on the ethanolic extract of Indian bee pollen to identify the presence of tannins, saponins, flavonoids, alkaloids, anthraquinones, terpenoids, glycosides, proteins, carbohydrates, resins, and acids (Kaur *et al.,* 2013; Sundarraju *et al*., 2014). The cold macerated ethanolic extract was subjected to GCMS analysis to identify the phytochemical constituents present in it. An autosampler system (7693) was equipped for the sample injection process. Helium was used as the carrier gas, with a post-run total flow of 2 minutes at 25 ml/min. The splitless flow rate was maintained at 1 ml/min, and the constant flow rate was maintained at 1 ml/min. An Agilent DB5MS capillary column of length 30m, an internal diameter 0.25mm, and a thickness 0.25 microns was used. The total run time was approximately 28 minutes, starting with an injector volume of 1µl. The pressure varied from 7.6522 psi, and the detector scan ranged between 50 and 500 with a 0.5s interval. The split ratio was 100:1, and the split flow was 1 ml/min. The acquired spectra of the chromatogram were cross-referenced with a mass spectral library (National Institute of Standards and Technology (NIST) version 14.0) to identify the eluted chemical compounds in the bee pollen extract using retention time and peak area.

***In silico* molecular docking:** Preparation of ligands: The phytochemical constituents, such as Hexadecanoic acid, Pentadecanoic acid,

Pyrroloindole and Alpha amyrin, were selected from the GC-MS chromatogram of the cold ethanolic bee pollen extract. Ro 48-8071 fumarate (an Oxidosqualene cyclase (OSC) inhibitor) was taken as the standard and compared with the binding energy of the phytochemicals. The 2D chemical structures of the chosen phytochemical compounds were retrieved from the PubChem database at the National Center for Biotechnology Information (NCBI) (https://pubchem.ncbi.nlm.nih.gov), and the 3D structure of the ligands was drawn using ChemSketch software. The ligands were prepared by energy minimization and the addition of hydrogen atoms using the ChemSketch software building tool. The 3D structures were saved in Protein Data Bank (PDB) format and prepared for the docking analysis using ChemSketch software. The protein molecular target was obtained from the protein database. The structure of human OSC (1W6J) was chosen for molecular docking studies based on literature reviews.

Cavity prediction and binding site analysis: Computed Atlas of Surface Topography of Proteins (CASTP), an online tool, is employed for the precise identification, delineation, and quantitative assessment of the geometric and topological attributes of target protein structures (Binkowski *et al*., 2003).

Visualization of target proteins and ligands: The atomic charges of the amino acid residues were fixed, and energy minimization was carried out. The prepared target protein structures, H-bond, and non-bond interactions of ligands with the active site residues were analyzed using the UCSF Chimera software to obtain high resolution images (Narayanaswamy *et al.,* 2014).

Docking: Geometrical optimization of the input compounds was performed using the Arguslab software to obtain a stable structure of the prepared compounds. After preparing the ligand and target protein structures, molecular docking was performed using Autodock 4.0 software. The standard operational protocol for ligand-protein (enzyme) docking was followed (Jemal, 2019).

RESULTS AND DISCUSSION

The qualitative phytochemical analysis showed presence of various bioactive compounds (*viz.,* acids, anthraquinone, alkaloids, carbohydrates, flavonoids, glycosides, phenol, proteins, quinone, resins, saponin, steroids, tannin and terpenoids) in the bee pollen extract. The results of GCMS with the standards of acquired chromatogram were compared with NIST and diverse compounds were recognized. A total number of 40 volatile phytochemical compounds were noted in the extract by considering retention time, peak number and peak area with percentage (Fig. 1). The initial peak detected was 1, 3, 5 - benzenetriol (1.02 %) at the time of 6.9 minutes. Other compounds such as 4HPyran-4-one, 2, 3 diHydro-3,5 dihydro 6methyl (1.93%), methyl salicylate (1.12 %), 4 mercaptophenol (9.89%), pyridine 2 fluro (9.89%), thiophene 2-propyl (9.89%), glucuronolactone (1.14%), 2-heptane isothiocyanate (1.14%), D mannoheptulose (0.71%), hexadecanoic acid (16.5%), pentadecanoic acid (4.34%), alpha amyrin (4.10%), pyrroloindole (4.10 %), oleic acid (1.23%), dodecanol (1.23%), taraxa sterol (2.23%), lupeol (2.23%), 9,12,15 octadecatrienoic acid (1.58%), and squalene (0.66%) were also detected. The ultimate peak was ethyl vanillin (0.85%) at the retention time of 26.5 minutes. These compounds (Table 1, 2) are reported for certain biological values (Aragão *et al*., 2006; Mujeeb et *al*., 2014; Manjal *et al*., 2019; Alam *et al*., 2021).

***In silico* molecular docking:** The five selected phytochemical constituents of cold macerated bee pollen extract were selected and their molecular structures were obtained from PubChem Database for chemical compounds at NCBI and the 3D structures were obtained from UC SF chimera (chimera). The selected active compounds of extraction were subjected to molecular *in silico* docking with an Oxidosqualene: Ianosterolcyclase enzyme to show the hypocholesterolemic activity of bee pollen. The protein structures of target enzyme Oxidosqualene: Ianosterolcyclase was obtained from Protein data bank and analysed using CASTP web server for the characterization of

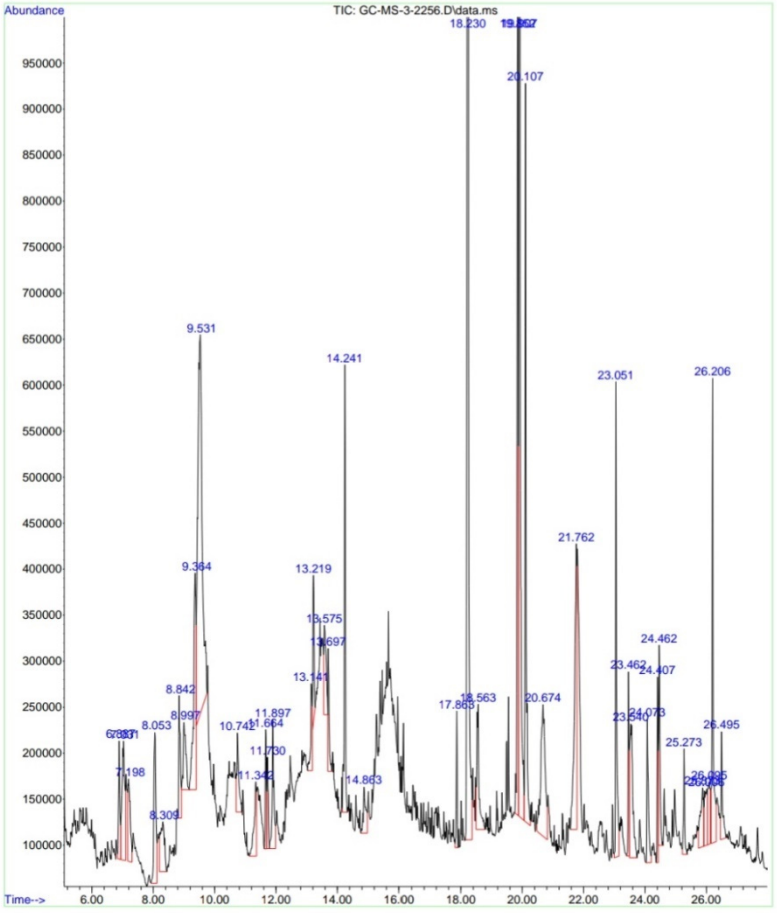

Fig. 1 GCMS Analysis of cold macerated ethanolic extract of bee pollen

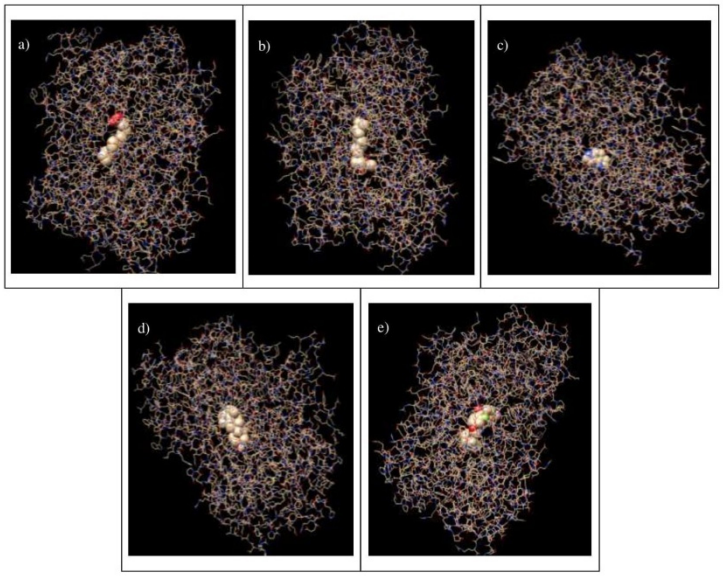

Fig. 2 Stick model image of docking of phytocompounds with Oxidosqualene: Ianosterolcyclase enzyme
a) hexadecanoic acid, b) Pentadecanoic Acid, c) Pyrroloindole, d) Alpha Amyrin, d) Ro 48-8071 fumarate

Table 1. GCMS analysis in cold macerated ethanol extract of bee pollen

No	Compound	Molecularformula	Mol.wt (g/mol)	Retention time (mins)	Peak area (%)
1	Hexadecanoic Acid	$C_{16}H_{32}O_2$	256.43	18.230	16.50
2	4 Mercaptophenol	C6H6OS	126.18	09.531	9.89
3	Pyridine, two fluro	C5H4FN	097.09	09.531	9.89
4	Thiophene 2-propyl	C7H10S	126.22	09.531	9.89
5	PentadecanoicAcid	C15H30O2	242.40	20.107	4.34
6	Alpha Amyrin	C30H50O	426.70	21.762	4.10
7	Pyrroloindole	C12H12N2	154.17	21.762	4.10
8	Lupeol	C30H50O	426.70	23.540	2.23
9	Taraxasterol	C3H50O	426.70	23.540	2.23
10	4HPyran-4-one, 2, 3diHydro-3,5 dihydro 6methyl	C6H8O4	144.12	08.053	1.93
11	9,12,15 octadecatrienoic acid	C18H30O2	278.40	24.642	1.58
12	Oleic Acid	C18H34O2	282.00	23.462	1.23
13	Dodecanol	C20H42O	186.33	23.462	1.23
14	Glucuronolactone	C6H8O6	176.12	13.574	1.14
15	Methyl Salicylate	C8H8O3	152.15	08.842	1.12
16	D Mannoheptulose	C7H14O7	210.18	14.863	0.71
17	Squalene	C30H50	410.70	25.273	0.66

active sites. The docking results of Oxidosqualene: Ianosterolcyclase and phytocompounds showed a significant interaction between ligand and target enzyme. All the compounds bound to the target enzyme with single hydrogen bond. The hexadecenoic acid interacted with histidine residue and pentadecanoic acid with tryptophan residue, pyrroloindole and alpha amyrin with aspartic acid residue and Ro 48-8071 fumarate with tyrosine residue of the target enzyme with a binding energy of -5.01 Kcal/mol, -6.55 Kcal/mol, -6.00 Kcal/mol, -13.37 Kcal/mol, and-10.21 Kcal/mol, respectively (Table 3, Fig. 2).

A commonly used technique for determining the botanical origin of pollen loads is the microscopic pollen analysis since, the size, shape and surface properties of pollen grains are characteristic to particular plant species (Kieliszek *et al*., 2018). Pollen is usually marketed after drying, but freezing and lyophilization are also acceptable techniques for preservation (Thakur and Nanda, 2020). Bee pollen is consumed as a food supplement for its varied health benefits. The technique of cold maceration is employed to obtain ethanolic extract of bee pollen and the total yield of cold macerated ethanolic bee pollen extraction was 5 per cent. Pollen extracts have been documented to show hypolipidemic activity, effectively reducing the levels of total lipids, triacylglycerol and cholesterol. Consequently, they exhibit potential effects to address cardiovascular diseases (Polanski *et al*., 1998). The GCMS analysis exhibits the presence of various compounds with their own retention time,

Table 2. Biological activity of the compounds present in the ethanolic extract of bee pollen

No	Name	Nature	Biological activity
1.	Hexadecanoic Acid	Saturated fatty acids	Anti-inflammatory, Anti-hypoxic Antipruritic, Antithrombotic, Antinociceptive, Antiparasitic
2.	4 Mercaptophenol	Phenol	Anti-cancer agents against melanoma and breast cancer cell lines
3.	Pyridine, two fluro	Inositol	Kinase 1 inhibitor, Useful in clinical oncology
4.	Thiophene 2-propyl	Hetro cyclic	Anti-microbial, Anti-cancer, Anti-inflammatory, Anti-hypertensive, Analgesic
5.	Pentadecanoic Acid	Saturated fatty Acids	Antibacterial
6.	Alpha Amyrin	Triterpene	Anti-tumor, anti-inflammatory, anxiolytic
7.	Pyrroloindole	Amine	Muscle relaxant, antifungal, antitumor, and antibiotic
8.	Lupeol	Triterpenoid	anti-inflammatory, anti-cancerous, cardioprotective, Anti-Diabetic, skin protective, antimicrobial agents, antiprotozoal agent, nephroprotective agent
9.	Taraxasterol	Triterpene	inflammatory diseases
10.	4H- Pyran-4-one, Hydro-3,5 dihydro, 6 methy	Flavonoid	Hyaluronic acid production, Melanin production 2,3 di linhibitor
11.	9, 12, 15 octadecatrienoic acid	Linolenic Acid	Anti-inflammatory, hypocholesterolemia cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, insectifuge
12.	Oleic Acid	Acids	antibacterial activity, antifungal activity
13.	Dodecanol	Long-chain fatty acids	antibacterial activity
14.	Glucuronolactone	Glucuronic acid	anti-inflammatory effect for the skin, and lowering abnormally high plasma concentrations of cholesterol
15.	Methyl Salicylate	Ester	Anti-inflammatory and analgesic agent
16.	D Mannoheptulose	Heptose	Breast cancer and to suppress the D-glucose induced insulin release
17.	Squalene	Triterpene	Antioxidant, Antitumor activities

Source: Aragão *et al*., 2006; Mujeeb *et al*., 2014; Manjal *et al*., 2019; Alam *et al*., 2021

area and concentration. These compounds were reported for their therapeutic efficiency. Hexadecanoic acid (16.5%) (saturated fatty acids) has potential anti-inflammatory, antiallopactic and antioxidant properties (Cupido *et al*., 2022). Mercaptophenol known for its anti-cancer properties against melanoma and breast cancer cell lines (Ruzza *et al*., 2009; Shpakovsky *et al*., 2014). Pyridine 2 fluoro (9.89%) is used as Kinase 1 inhibitor and in clinical oncology (Laha Roy *et al*.,

Table 3. *In silico* molecular docking analysis in the cold macerated extract of bee pollen

No	Compounds	Binding energy (Kcal/mol)	Amino acid interaction residues
1.	Hexadecanoic acid	-5.01	1W6J:A:HIS232:ND2—O:UNL1
2.	Pentadecanoic Acid	-6.55	1W6J:A:TRP581:O—O:UNL1
3.	Pyrroloindole	-6.00	1W6J:A:ASP455:OD2—O:UNL1
4.	Alpha Amyrin	-13.37	1W6J:A:ASP455:OD2——H:UNL1
5.	Ro 48-8071 fumarate	-10.21	1W6J:A:TYR704:OH——C:UNL1

Note: No. of Hydrogen is one in all compound bonds

2018). Thiophene derivatives show antimicrobial (Tehranchian *et al*., 2005), analgesic and anti-inflammatory (Pillai *et al.,* 2005), antihypertensive (Russell *et al*., 1988), and antitumor activity (Chen *et al*., 2015).

In animals, the OSC enzyme is primarily produced and catalyzed with the help of lanosterol, which is involved in the cholesterol biosynthesis pathway. Cholesterol is crucial for body temperature regulation and is a precursor for testosterone in males and oestradiol in females (Liang *et al*., 2014). In this study, the docking results showed that the active compounds in the ethanolic extract of bee pollen strongly bound with the target OSC enzyme. The binding interaction between alpha amyrin and OSC enzyme was comparatively higher than other compounds, including standard drugs. Alpha amyrin is one of the effective compounds with antioxidant, anti-inflammatory and hypoglycemic properties (Gunnam and Nangia, 2019), and the binding energy of the alpha amyrin is high (-13). Ro 48-8071 fumarate acts as an inhibitor of OSC with IC_{50} of approximately 6.5nM (Mallick and Dighe, 2014). Ro 48-8071 fumarate has a binding energy of -10.21 which is lower than alpha amyrin. Ro 48-8071 fumarate is a 2, 3-Oxidosqualene cyclase (OSC) inhibitor (IC_{50}= 6.5 nM); blocks cholesterol synthesis in HepG2 cells (Morand *et al.,* 1997). Since it can withstand the enzyme for a longer duration of time, all these four compounds are present in high percentage in the bee pollen ethanolic extract. These compounds significantly interact with the OSC enzyme and serve the enzyme inhibitory action. Targeting the OSC enzyme to reduce cholesterol is an alternative way to discover a new hypercholesteroemic drug. This *in silico* molecular docking study showed the efficiency of compounds present in the bee pollen extract in inhibiting cholesterol biosynthesis through OSC inhibition.

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