"DESIGN AND DEVELOPMENT OF VALUE ADDED APICEUTICAL PRODUCTS"

A THESIS SUBMITTED TO
BHARATI VIDYAPEETH UNIVERSITY, PUNE
FOR AWARD OF DEGREE OF
DOCTOR OF PHILOSOPHY IN PHARMACOGNOSY
UNDER THE FACULTY OF PHARMACEUTICAL SCIENCES

SUBMITTED BY

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UNDER THE GUIDANCE OF

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JULY 2015

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "Design and Development of Value Added Apiceutical Products" for the degree of 'Doctor of Philosophy' in the subject of Pharmacognosy under the faculty of Pharmaceutical Sciences has been carried out by Mr. Sameer S. Ketkar in the Department of Pharmacognosy, at Bharati Vidyapeeth Deemed University's Poona College of Pharmacy, Pune during the period from August 2010 to July 2015 under the guidance of Prof. K. R. Mahadik, Principal, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune.

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CERTIFICATE

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled "Design and Development of Value Added Apiceutical Products" submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the degree of Doctor of Philosophy (Ph.D.) in Pharmacognosy under the faculty of Pharmaceutical Sciences is original piece of work carried out by me under the supervision of Prof. K. R. Mahadik, Principal, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune. I further declare that it has not been submitted to this or any other University or Institution for the award of any degree or Diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune

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UNIVERSITY of BRADFORD
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Professor and Director

This is to certify that Mr. Sameer Shridhar Ketkar, has been a visiting researcher at Centre for Pharmaceutical Engineering Science, Bradford, UK under the UKIERI (UK-India Education and Research Innovative) project entitled “Process analytics enabled green technologies for processing of poorly soluble drugs” funded by British Council, India. Mr. Sameer visited Centre for Pharmaceutical Engineering Science, Bradford during July 2013 to October 2013. During this tenure Mr. Sameer worked on development of pharmaceutical cocrystals for poorly soluble actives. Further he also worked on development of green processing methods for natural products and investigation of dielectric properties of different systems.

During said tenure, Mrs Sameer worked under my guidance and to my satisfaction. He acquired knowledge and hands on training in different aspects of green crystal engineering technique for development of pharmaceutical cocrystals. Further he also worked on microwave processing of pharmaceuticals as well as natural products. During this tenure we found him sincere with good conduct.

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Dedicated to honeybees

who gather and spread around

the Goodness of Nature ….

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Date: **SAMEER S. KETKAR**

AT A GLANCE:

Apiculture, the science and practice of honeybee keeping contributes to the field of nutrition and medicine by providing access to 'apiproducts' such as honey, propolis, bee pollen, royal jelly, etc, which demonstrate several beneficial effects in health care. Apart from traditional apitherapy, recent scientific explorations in the field of apiproducts are drawing major attention from researchers and nutraceutical industries worldwide.

Despite of diverse therapeutic potential of apiproducts, the poor solubility (bee pollen, propolis); waxy or resinous nature (propolis, beeswax); sticky consistency (honey, propolis) and physical instability (royal jelly) impart hurdles in their processing and formulation development. Further large amount of scientific evidences dictate poor digestibility and bioavailability of apiproducts such as bee pollen and propolis. Most of such apiproducts cannot be consumed in the state in which they are produced by bees, however their nutritional enrichment and potentiation of therapeutic efficiency necessitates effective processing of raw apiproducts.

Present research work was initiated with the aim to achieve nutritional enrichment and therapeutic potentiation of apiproducts. Accordingly, the research work investigates applicability of pharmaceutically acceptable excipients for processing of apiproducts converting them into fortified "Value Added Apiceuticals" with improved therapeutic efficiency. It is also objective of this research to develop mechanistic understanding in various aspects of innovative extraction processes to be used for apiproducts. The work further involves fundamental studies on active moiety originating from apiproducts in view of modulating its physicochemical parameters which directs improvement in biopharmaceutical performance.

Present dissertation categorizes the research work in two parts:

A) Multicomponent systems:

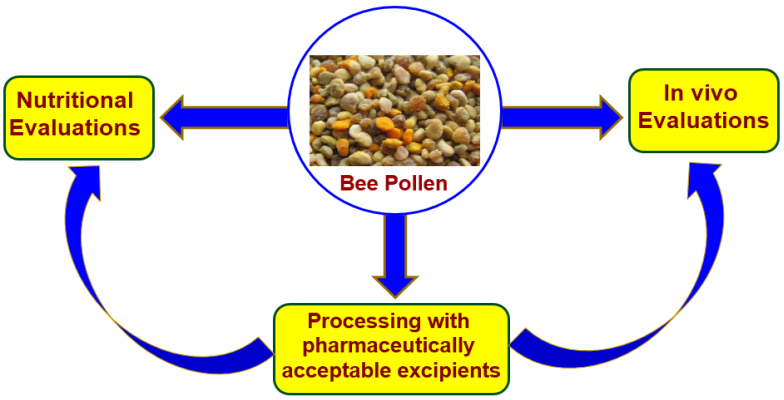
These include studies on bee pollen and propolis (as a whole and in the form of extracts thereof) in view of nutritional enrichment using pharmaceutically acceptable excipients.

B) Single component system:

This include studies on therapeutically active polyphenolic constituent of propolis i.e. Caffeic acid phenethyl ester (CAPE), in view of modulating its physicochemical properties and improving the aqueous solubility.

I. Bee Pollen:

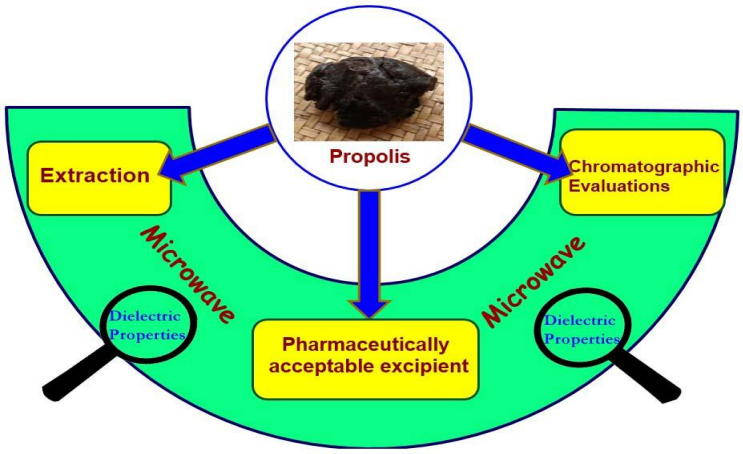
Studies were undertaken for exploration and potentiation of nutraceutical competence of monofloral Indian mustard bee pollen (MIMBP). Part A of the study analyzes nutraceutical candidature of MIMBP in terms of nutritional and chemical composition. Part B explores processing of MIMBP using edible lipid surfactant mixtures, converting them into potentiated pollen with improved polyphenols availability. Further preclinical studies were undertaken to investigate the effect of neat and processed bee pollen on exercise induced oxidative stress muscular implications in Wistar rats. The studies established muscle protectant role of bee pollen. Further the processing of MIMBP with edible lipid-surfactant mixture was found to improve its therapeutic efficiency.



Schematic representation of studies on bee pollen

II. Propolis:

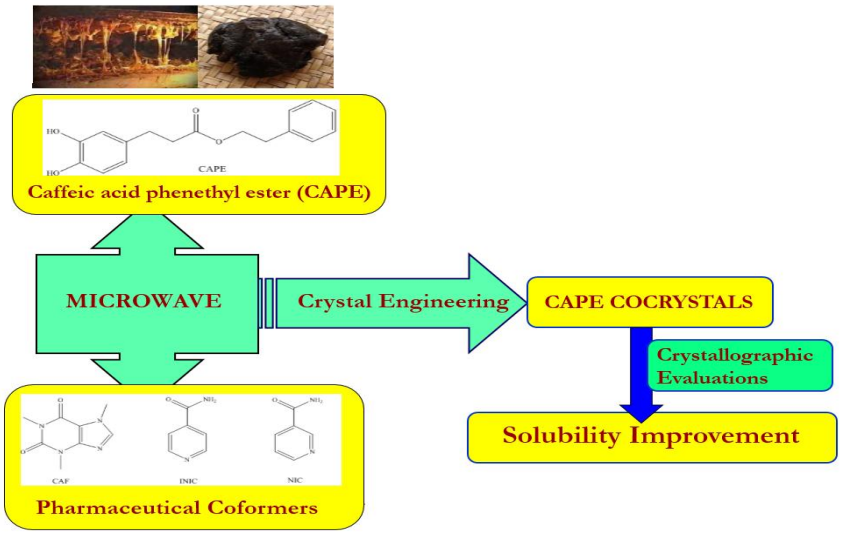
Proposed herein a green approach for improving microwave assisted extraction (MAE) of bioactive flavonoids from propolis using pharmaceutically acceptable and biocompatible excipient (Gelucire 44/14) as extractant. Part A of the study describes development and validation of HPLC-PDA method for simultaneous estimation of principal flavonoid marker components of propolis. Part B describes studies on microwave assisted extraction of propolis using Gelucire 44/14 as an exractant in comparison with conventional MAE. Further efforts were made to understand the mechanism of Gelucire 44/14 based MAE in view of dielectric properties of the solutions used. The study demonstrated effectiveness of Gelucire 44/14 in improving the microwave assisted extraction of flavonoids from propolis by modulating dielectric properties of water.



Schematic representation of studies on propolis.

III. Caffeic acid phenethyl ester (CAPE)

Studies were performed on therapeutically active polyphenolic constituent of propolis i.e. Caffeic acid phenethyl ester (CAPE), in view of modulating its solid state properties and improving its aqueous solubility. The study involves application of crystal engineering technique (microwave-assisted cocrystallization) to generate CAPE cocrystals using pharmaceutically acceptable coformers. The generated cocrystals were characterized using diffraction and spectroscopic tools. The study demonstrated successful development of CAPE cocrystals [CAPE-caffeine, CAPE-isonicotinamide and CAPE-nicotinamide] with improved aqueous solubility as compared to the parent compound.



Schematic representation of study on CAPE

Index

List of Tables:

List of Figures:

ABBREVIATIONS

MIMBP: Monofloral Indian mustard bee pollen

PMIMBP: Processed monofloral Indian mustard bee pollen

GAE: Gallic Acid Equivalent

QE: Quercetin Equivalent

P: Polar axis

E: Equatorial diameter

CL: Colpus length

HPLC-PDA: High performance liquid chromatography-Photo diode array

Rt: Retention time

SOD: Superoxide dismutase

GSH: Glutathione

MDA: Malonaldehyde

NO: Nitric oxide

RT PCR: Reverse transcriptase polymerase chain reaction

MCT: Medium chain triglyceride

MAE: Microwave Assisted Extraction

MW-EEP: Ethanolic extract of propolis (microwave assisted)

MW-GP: Gelucire 44/14 based propolis extracts (microwave assisted)

ε': Dielectric constant

ε": Dielectric loss

Tan δ: Dissipation factor

DSC: Differential scanning calorimetry

CAPE: Caffeic acid phenethyl ester

API: Active Pharmaceutical Ingredient

GRAS: Generally Regarded as Safe

PXRD: Powder X-ray Diffraction

CAF: Caffeine

NIC: Nicotinamide

INC: Isonicotinamide

Chapter 1. Introduction

This chapter provides a brief background about apitherapy and different apiproducts discussed in view of their chemical composition and beneficial applications. The regulatory categorization and global market scenario of apiproducts in different countries has been discussed in comparison with Indian perspective. This is followed by genesis of current work and the literature review based on bee pollen, propolis and its active polyphenolic constituent CAPE. Further the literature revealed need of work has been addressed.

1.1. Background:

1.1.1. Apitherapy and Apiproducts:

'Apis' is a Latin word for bee. "Apitherapy" or "bee-therapy" is a popular science of treatment using bees and their products to prevent, heal or recover from several ailments benefiting the mankind as well as animal kingdom (Gupta and Stangaciu, 2014). Worldwide the practice of apitherapy has been followed by alternative health practitioners since traditions. The apitherapy possesses ancient origins. Records from ancient India and Egypt display use of honey in treating wounds. Several religious texts and traditional systems of medicines refer to honey and its healing powers. According to a report, first identified prescription about honey was written on clay tablet found in the Euphrates valley between 2100 and 2000 BC (Bogdanov et al., 2008). The father of medicine and famous Greek physician Hippocrates documented the physical effects of honey as: "It causes heat, cleans sores and ulcers, softens hard ulcers of the lips, and heals carbuncles and running sores". The Sanskrit Veda of ancient India, the Bible, the Koran have reported honey as a remedy for many disorders (Bogdanov et al., 2008).

The Apimondia Standing Commission for Apitherapy of International Federation of Beekeepers' Associations defined 'Apitherapy' as a medical concept, based on scientific foundations corroborating traditional knowledge, including: A) Bee production procedures aimed at medical development; B) Transformation of hive product procedures, alone, or in association with medicinal plants and their derivatives (api-pharmacopoeia); and C) Clinical protocols incorporating use of the api-pharmacopoeia and/or of the bees (api-medicine) (Bradbear, 2009)

1.1.2. Genesis of apiproducts by honeybees:

Honeybees produce honey, propolis, royal jelly (bee milk), pollen, beeswax and bee venom as the main apiproducts in their hives. They collect nutrients from vegetation, process them by addition of own saliva, stomach fluids and gland secretions,

allow them to ripen inside the hives. Thus honeybees perform unique transformations of the raw material provided by plant sources into products like propolis, honey and bee pollen converting the plant pharmacology into array of 'apiproducts', which provide enhanced functional support in health care. Figure 1.1 denotes schematic representation of transformation pathway of collected raw materials into 'apiproducts' inside the bee hives.

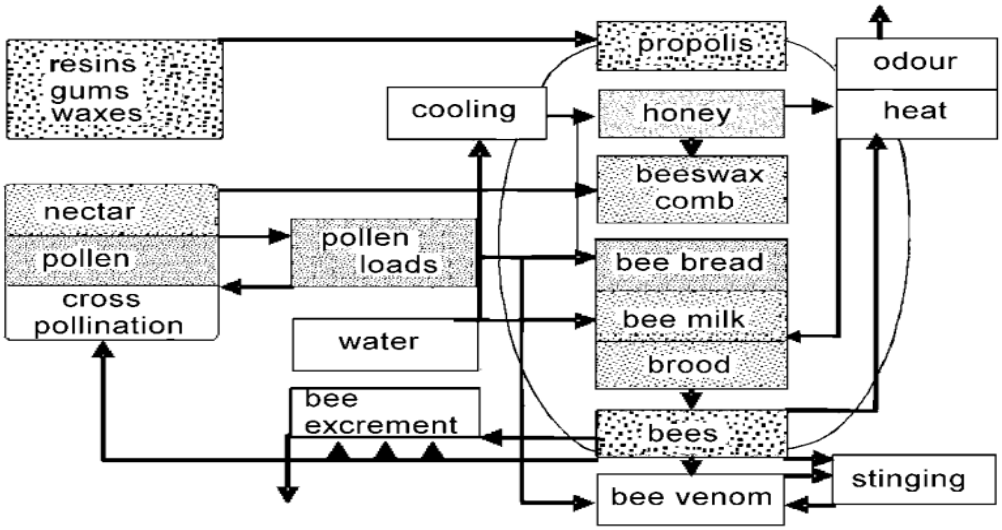


Figure 1.1. Genesis of apiproducts by honeybees from nutrients collected from vegetation. [Source:(Mutsaers et al., 2005)]

Honeybees gather nectar from plant sources, make honey out of it and store at the top and outer walls of the hive as food supply or raw material for wax and heat generation. Bees produce beeswax in their wax glands by using stored honey as raw material and energy source. During nectar collection, pollen from flower stamens stick to the chest hairs of bees. Bees comb the pollen off and roll them into pellets by using hind legs. Landing of these bees on several flowers of one species induce cross-pollination and fructification of pollen. The pollen loads are carried to the honeycomb cells where they are ripened into bee bread. Young bees utilize secretions from own glands to process the bee bread into bee milk and royal jelly, formed along with the eggs and pupae (brood). The brood is situated in the middle of colony. Bees emerging from brood may

be queens, worker bees or drones (males). Worker bees produce honey from nectar, bee bread from pollen, and clean the cells using propolis. They also produce bee milk and royal jelly from bee bread. Worker bees and the queen produce bee venom in venom glands and store it in the venom sac. The forager bees collect nectar, pollen, gums, resins and waxes from trees and plants. The gums, resins and waxes are mixed by adding saliva to produce propolis. Heat and warm air released from a hive (bee odor) is known to possess therapeutic value. Water is stored in bee stomach contributes to high moisture content of bee milk and brood. Table 1.1 displays information about the collection, transportation and processing of raw materials into the apiproducts by honeybees.

Table 1.1. Raw materials transforming into bee products inside the hive

Vegetation		Inside the hive		
Substance	How bees transport them	Processing into.	Location	Function
Pollen	In pollen baskets on the hind legs	Bee bread Bee milk Brood	In lower part of combs, In brood cells with larvae In centre of brood	Food Food Food development
	On the breast hairs	--	Outside the hive	Pollination
Nectar	Upper aero digestive tract of honeybee	Honey	On top and outer combs of hive	Food, Raw material for wax and warmth
		Wax	In the form of comb	Building of combs Nest for brood and food
Water	Upper aero digestive tract of honeybee	Evaporation	Honey stomach of bees	Cooling Production of bee milk
Gum Resin Wax	On legs	Propolis	Wall of hive	Hive wall putty Heat regulation Cleaning of cells

[Source: (Mutsaers et al., 2005)]

For preservation, consumption and marketing purposes (post harvesting), the beekeepers process the products to increase their acceptability and market value.

1.1.3. Apiproducts: Composition and Beneficial Applications:

Honey, propolis, royal jelly (bee milk), bee pollen, beeswax and bee venom are the six important apiproducts, popularly known as "Bee hexagon", which possess extraordinary medicinal and commercial values. Literature for bee products reveals honey, propolis and bee venom have been explored for medicinal applications. Pollen and royal jelly find mainly nutrient and dietary applications whereas bees wax confers exploration for cosmetic and non-medicinal uses. (Figure 1.2)

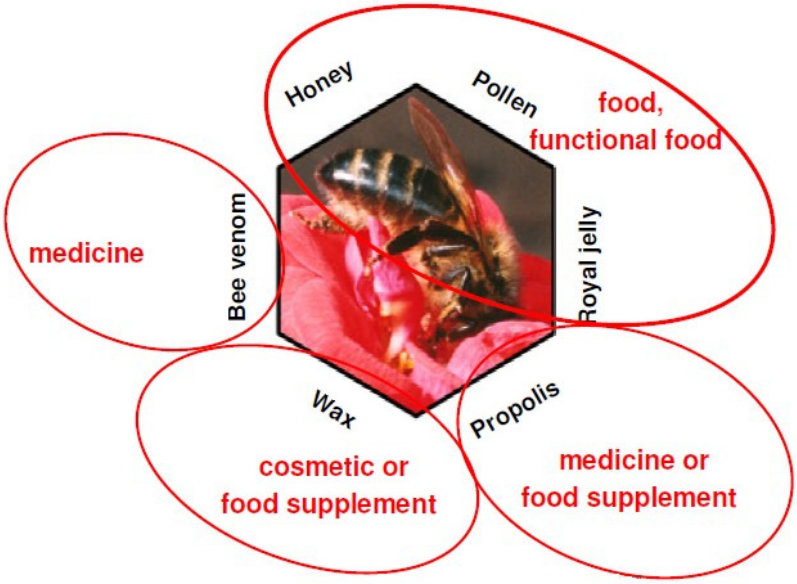


Figure 1.2. Bee products and their potential applications
[Source: Bee product Science, www.bee-hexagon.net]

1.1.3A. HONEY:

Honey the most explored apiproduct from medicinal as well as economic point of view worldwide. It has been widely adopted as food and medicine by both ancient and modern generations. Honey is a viscous, sweet and palatable liquid with high nutritional value and array of health benefits (Bogdanov et al., 2008). Honeybees produce 'blossom honey' by secreting nectars of flowers, and 'honeydew honey' or 'forest

honey' by secreting the exudates of plant sucking insects (Aphids) (Ajibola et al., 2012).Chemically honey comprises of sugars and water along with vitamins, amino acids, proteins, polyphenols, minerals, micronutrients and aroma compound (White and Doner, 1980, Bogdanov et al., 2008). It is a rich source of carbohydrates especially monosaccharides fructose and glucose providing more energy than artificial sweeteners (Bradbear, 2009, Ajibola et al., 2012). Table 1.2 denotes nutritional composition of blossom honey and honeydew honey.

Table 1.2. Nutritional composition of honey

Composition	Blossom Honey (g/100g)	Honeydew Honey (g/100g)
Water	15-20	15-20
Total sugars	79.7	80.5
Monosaccharides		
Fructose	30-45	28-40
Glucose	24-40	19-32
Diasaccharides		
Sucrose	0.1-4.8	0.1-4.7
Others	2.0-8.0	1.0-6.0
Trisaccharides		
Erlose	0.5-6.0	0.1-6.0
Melezitose	<0.1	0.3-22
Others	0.5-1.0	0.1-6.0
Minerals	0.1-0.5	0.6-2.0
Amino acids, proteins	0.2-0.4	0.4-0.7
Other acids	0.2-0.8	0.8-1.5
pH Value	3.2-4.5	4.5-6.5

[Source: (Ajibola et al., 2012)]

Honey also comprises range of vitamins such as ascorbic acid, pantothenic acid, niacin and riboflavin. The mineral composition of honey includes calcium, iron, magnesium, manganese, copper, phosphorus, potassium, zinc along with traces of selenium and chromium. The nutritional richness of honey made it food of choice. 70 –95 g/day is the prescribed dose of honey to achieve desirable nutritional and health benefits (Bogdanov et al., 2008, Ajibola et al., 2012). Honey also comprises several polyphenols including

flavonoid and carotenoid constituents conferring high antioxidant potential(Carlos et al., 2011).

Several preclinical and clinical reports document medicinal importance of honey in terms of antioxidant, immunomodulatory, antianaemic, anti-inflammatory, antibacterial, antifungal, antiviral, antiulcer, gastroprotective and cardioprotective activities (Ajibola et al., 2012, Eteraf-Oskouei and Najafi, 2013, Ediriweera and Premarathna, 2012, Escuredo et al., 2013)

Natural honey is documented to exhibit inhibitory effect against several species of bacteria including aerobes, anaerobes, gram-positives and gram-negatives (Eteraf-Oskouei and Najafi, 2013). It is also reported to be anti-infective against infections caused by *Staphylococcus aureus, Bacillus anthracis, Streptococcus faecalis, Salmonella typhi, Sal. Diarrhea, Pseudomonas aeruginosa Klebsiella pneumoniae, Mycobacterium tuberculosis, Corynebacterium diptheriae, Haemophilus influenza, Proteus species, Acinetobacter spp, Serratia marcescens,* etc (Jeffrey and Echazarreta, 1996, Ajibola et al., 2012).

Honey exhibits antifungal action against several species such as *Aspergillus, Penicillium, dermatophytes* (Brady et al., 1996, Sampath Kumar et al., 2010). Honey is also reported to exhibit antimicrobial activity against methicillin-resistant *Staph. aureus* (MRSA), (Maeda et al., 2008) and *H. pylori* infections (Al Somal N et al., 1994). Few reports disclose antiviral effects of honey against herpes lesions, rubella virus, etc (Bogdanov et al., 2008).

Plethora of reports document wound healing activity of honey. Honey exerts cleansing action on wounds, induces regeneration of tissues and reduces inflammation (Efem, 1988, Medhi et al., 2008). Several clinical trials document effectiveness of honey dressings in the treatment of burns (Meda et al., 2004).On account of these properties honey has been a choice of ingredient in adhesive or non-adhesive tissue dressings. Honey reduces the activities of cyclooxygenase1 and 2 thereby exhibit anti-inflammatory effects (Nasuti et al., 2006).

Several studies document efficiency of honey in the treatment and prevention of gastrointestinal tract diseases such as gastritis, duodenitis and gastric ulceration (Haffejee and Moosa, 1985, Al Somal N. et al., 1994, Gharzouli et al., 2002, Nasuti et al., 2006).

On account of rich polyphenolic and flavonoids content honey confers strong antioxidant, anticarcinogenic, antiatherogenic, immune modulating, antithrombotic and analgesic activities, (Frankel et al., 1998, Gheldof et al., 2002, Gheldof and Engeseth, 2002, Ajibola et al., 2012). Further it has been proved to exert protective effects in coronary heart diseases (CHD) and cardio vascular implications. Natural honey has been documented to exhibit antineoplastic activity in experimental bladder cancer (Swellam et al., 2003).

1.1.3B. BEE POLLEN:

Bee pollen is an apiproduct produced by honeybees *Apis mellifera* by gathering floral pollen grains, mixed with plant nectar and bee saliva for feeding its larvae during early stages of development. Pollen comprises male reproductive cells (gametophytes) gathered by honeybees from flower stamens (Krell, 1996, Campos et al., 1997a). During visiting flowers, honeybees approach the stamens where their bodies get covered with pollen. The bees compress pollen dust with their hind legs into the corbicula or pollen baskets on rear legs, moisten them by addition of mouth secretions and plant nectar to form pollen pellet. These pellets adhered to the hind legs of bee are carried to the hive where they are stored in honeycomb cells (Cocan et al., 2005, Margaoan et al., 2010). These are used as raw material to produce bee bread inside the bee hive by process of anaerobic fermentation (Komosinska V et al., 2015). Floral constancy is a unique behavior observed in honeybees which imparts uniform composition of single floral pollen in the gathered pollen baskets. The pollen basket, thus comprises pollen from single plant at a collection trip. Similarly honeybees gather pollen from many different plant species (Amaya MM, 2009, Komosinska V et al., 2015).

Bee pollen is documented to comprise more than 250 substances including proteins, amino acids, polyphenols, flavonoids, sugars, fatty acids, minerals, vitamins, macro and micro nutrients which make it an apiproduct of human interest (Campos et al., 1997b, Campos et al., 2010, Komosinska V et al., 2015).The composition of bee pollen varies with plant source and geographic origin. Literature revealed average composition for bee pollen belonging to different biological source and geographical origin as noted in table 1.3 mentioned below

Table 1.3. Composition of bee pollen

Component	**Composition (g/ 100g)**
Carbohydrates (fructose, glucose, sucrose, fibers)	13-55
Crude fibers	0.3 – 20
Protein	10-40
Fat	1-13
Vitamins	**mg in 100g**
Ascorbic acid (C)	7 – 56
b-Carotin (provitamine A)	1-20
Tocopherol (vitamine E)	4-32
Niacin (B3)	4-11
Pyridoxin (B6)	0.2- 0.7
Thiamin (B1)	0.6-1.3
Riboflavin (B2)	0.6-1.2
Pantothenic acid	0.5-2
Folic acid	0.3-1
Biotin (H)	0.05-0.07
Minerals	
Potassium (K)	400- 2000
Phosphor (P)	80-600
Calcium (Ca)	20-300
Magnesium (Mg)	20-300
Zink (Zn)	3-25
Manganese (Mn)	2-11
Iron (Fe)	1.1-17
Copper (Cu	0.2-1.6

[Source: (Campos et al., 2008)]

Apart from the above constitution of bee pollen, large number of scientific reports document richness of bee pollen from different geographical origin in their antioxidant

principles especially the polyphenols and flavonoids. The phenolic or flavonoid composition has been suggested as a quality parameter for assessment of bee pollen (Campos et al., 1997b). Worldwide several authors have analyzed the polyphenols content of bee pollen from different geographical origins. Table 1.4 compiles the documented ranges for polyphenols and flavoniods content of bee pollen from different geographical regions:

Table 1.4. Documented polyphenols and flavoniods content for bee pollen from different geographical origins:[Source: (Ketkar et al., 2014)]

Geographical Region	Total polyphenols content (mg/g GAE)	Total flavonoids content (mg/ g GAE QE)
Alagoas	360 to 810	--
Algeria	2190 to 2 668	571 to 1 457
Bahia, Brazil	4150 to 21 320	--
Parana state	660 to 1 090	--
Poland	1515 to 8 025	--
Portugal	1290 to 1 980	4500 to 7100 CE
Romania	2167 to 2 880	545 to 2 044
Spain	850 to 1 460	380 to 760
Southern Brazil	3046 ± 8.22	892 ± 55
Sonoran Desert US	1591 to 3 485	266 to 548
Transylvania	376 to 887	255 to 629

GAE: gallic acid equivalents; QE: quercetin equivalents; CE: catechin equivalents

Broadly, bee pollen is considered as functional food with array of nutritional and therapeutic benefits. On account of myriads of chemical composition these are reported to be “only perfectly complete food” (Feas et al., 2012) possessing wide array of pharmacological activities including antioxidant, anti-inflammatory, anti-allergic, antibacterial, immunomodulatory, hypolipidemic, hepatoprotective and detoxifying activities, etc. Detailed literature about the activity profile of bee pollen will be discussed in 1.3.1 section.

1.1.3C. PROPOLIS:

Propolis is an apiproduct of complex resinous nature, collected and harvested by honeybees especially *Apis mellifera* from variety of plant sources including cracks in

bark and leaf buds. Propolis, also known as 'bee glue' is a strongly adhesive material used by bees for construction, maintenance and protection of their hives. Bees generate propolis as a means of defense against intruders such as microbes, moulds that may enter into the hive preventing their putrefaction (Ghisalberti, 1979, Bankova et al., 2000). Literature reveals that propolis components may originate from three distinctive sources as: (a) 'vegetal' which includes plant exudates, resins secreted by buds of birch, chestnut, pine, maple, poplar, and lipid substances from plant wounds, gums and resins wounds; (b) 'animal' which includes materials secreted by bees such as saliva, wax; and (c) incidental substances (pollen, honey) mixed during propolis production (Marghitas et al., 2013). Propolis may differ in appearance and color varying from golden yellow, red, green or dark brown based on the plant source. It exhibits lipophilic nature, hard and sticky consistency at 25 to 45 ˚C, however becomes brittle when subjected to freezing(Krell, 1996, Wagh, 2013).

Crude propolis comprises complex chemical composition including resins (about 40-50%), waxes (about 25-30%), essential oils (about 10%), pollen (5%) and several organic compounds (5%)(Burdock, 1998, Park et al., 2002, Naik et al., 2013b). It is reported to comprise more than about 300 constituents with diverse pharmacological activities. Several researchers have explored polyphenols especially flavonoids and phenolic acids (benzoic acid derivatives, cinnamic acid derivatives) as therapeutically active constituents of propolis. Different phenolic acids and flavonoids documented to be present in propolis include Caffeic acid, p-Coumaric acid, Ferulic acid, Isoferulic acid, 3,4-Dimethyl-caffeic acid (DMCA), Cinnamic acid, Quercetin, Quercetin methyl ethers, rutin, Pinocembrin, Pinobanksin, Pinobanksin methyl ethers, Pinobanksin ester derivatives, Apigenin, Kaempferol, Chrysin, Methoxy-chrysin, Galangin, Isorhamnetin, Luteolin-methyl-ether, Cinnamylidene acetic acid, Caffeic acid ester derivatives [eg: Caffeic acid phenylethyl ester (CAPE)], p-Coumaric ester derivatives [eg: p-Coumaric prenyl ester, p-Coumaric cinnamyl ester], p-Methoxy cinnamic acid cinnamyl ester, etc.(Park et al., 2002, Bankova, 2009, Pellati et al., 2013).

Presence of sugars, minerals, amino acids and heteroaromatic constituents has been documented in propolis. Further it is also reported to contain volatile constituents such as sesquiterpene alcohols, etc (Xu et al., 2009b, Bankova et al., 2014b). Literature disclosing chemical composition of propolis from different geographical regions show characteristic classes of compounds correlated with specific plant sources in the relevant geographical origins (Salomao et al., 2004, Marghitas et al., 2013). European propolis majorly comprises typical poplar (*populous nigra*) bud phenolics (flavonoids, phenolic acids and esters). Brazilian propolis mainly comprises leaf resin of *Baccharis dracunculifolia* which mainly contains prenylated derivatives of p-coumaric acid, acetophenone. Cuban propolis mainly comprises poly-isoprenylated benzophenones (Bankova, 2005).

Among the several active flavonoids present in propolis, Caffeic acid phenylethyl ester (CAPE) has received major attention of research on account of array of biological activities ascribed to it (Tolba et al., 2013). On account of diverse chemical composition comprising polyphenols especially the CAPE, propolis exhibits wide range of pharmacological activities including antioxidant, antibacterial, antifungal, antiviral, hepatoprotective, antimutagenic, cytotoxic, antiproliferative, immunomodulatory, analgesic, anti-inflammatory, antidiabetic, cardioprotective and neuroprotective activities. (Burdock, 1998, Banskota et al., 2001, Bankova, 2005, Wagh, 2013, Marghitas et al., 2013). Detailed literature about the activity profile of propolis will be discussed in section 1.3.2.

1.1.3D. ROYAL JELLY:

Royal jelly is a thick, milky secretion of honeybee used as a nutrient source for larvae and adult queens. It is a secretion product of hypopharyngeal and mandibular glands of worker bees and makes vital part of larvae diet (Ramadan and Al-Ghamdi, 2012). It plays immense role in growth, maturation process and caste differentiation of honeybees(Pavel et al., 2011). Royal jelly is known to be beneficial remedy for humans

and has been used in folk medicines. It is a whitish-yellow viscous jelly substance soluble in water. It exhibits pungent odor, sweet and sour taste.

Chemically royal jelly comprises water (60–70%), protein (12–15%), carbohydrate (10–16%), lipid (3–7%), traces of mineral salts and vitamins (Ramadan and Al-Ghamdi, 2012). It also contains 17 amino acids including 8 essential ones along with amino and gamma globulins, aspartic acid. Royalactin, a protein component also known as Major royal jelly protein (MRJP1) is reported to exert key part in development of honeybee queen (Kamakura, 2011). Trans-10-hydroxy decanoic acid, also called as Royal jelly acid has been reported to be a potent antimicrobial agent (Sugiyama et al., 2012). Royal jelly is known to be natural source of pure acetylcholine.

On account of its complex chemical composition, royal jelly has been reported with variety of functional properties such as antioxidant, vasodilator, hypotensive, neurotrophic, hipocholesterolemiant, hipoglicemiant effects. Further it has been documented to possess hepatoprotective, anti-inflammatory, immunomodulatory, anti-allergic, antibiotic, disinfectant and antitumor activities (Viuda MM et al., 2008, Ramadan and Al-Ghamdi, 2012, Li et al., 2013).

Several reports document antioxidant effects of royal jelly in different *in vitro* and *in vivo* models (El-Nekeety et al., 2007, Silici et al., 2009). It also exerts protective effects on tissue DNA against oxidative damage conditions (Inoue et al., 2003). Royal Jelly demonstrated protective effect against radiation induced oxidative stress in rats (Cihan et al., 2013).

Royal jelly has been reported to exhibit potent antibacterial effect ascribed to royalisin protein (Fujiwara et al., 1990). It has been documented to be effective in treating mucositis in hamsters (Suemaru et al., 2008). Peptide composition of royal jelly is known to exert antihypertensive effects in rats (Tokunaga et al., 2004).

The fatty acid components of royal jelly have been proved to modify the dendritic cell mediated immune reaction thereby exhibiting immunomodulatroy effects (Gasic et al., 2007). Royal jelly also boosts collagen synthesis (Koya M et al., 2004).

The lipid constituents of Royal jelly especially the fatty acid Trans-10-hydroxy decanoic acid and its derivatives have shown potent antitumor activity (Townsend et al., 1960). It has displayed neuroprotective and neurotrophic effects on hippocampus mouse brain (Pavel et al., 2011).

1.1.3E. BEE VENOM:

Bee venom is a transparent, odorless liquid with ornamental pungent smell and bitter taste. Venom glands accompanying the sting apparatus of worker and queen bees produce the bee venom and store it in the venom reservoir. The sting apparatus injects the venom during stinging process (Schmidt and Buchmann, 1992). The honeybee venom majorly comprises proteins and peptides along with other low molecular weight components. It is reported to contain 88% water. The major components of dry bee venom is summarized below (Table1.5)

Table 1.5. Composition of dry bee venom

Class of molecules	Components	% of dry venom
Proteins (Enzymes)	Phospholipase A2	10-12
	Hyaluronidase acid	1-3
	Phosphomonoesterase	1.0
	Lysophospholipase	1.0
	ά-glucosidase	0.6
Peptides	Melittin	40-50
	Apamine	1-3
	Mast Cell Degranulating Peptide (MCD)	1-2
	Secapin	0.5-2
	Procamine	1-4
	Adolapin	1
	Protease inhibitor	0.8
	Tertiapinc	0.1
	Small peptides (with less than 5 amino acids)	13-15
Active amines	Histamine	0.5-2.0
	Dopamine	0.1-0.2
	Noradrenaline	0.1-0.7
Amino Acids	ά -aminobutyric acid	0.4-0.5

	ά -amino acids	1
Sugars	Glucose and fructose	2
Other	Phospholipids	5
	Volatile compounds (ether)	4-8
	Minerals (Ca, P, Mg)	3-4

[Source: (Ali, 2012)]

Bee venom therapy is popular since ancient times and involves administration of bee stings, venom, acupuncture and injection (Munstedt et al., 2005). Several reports demonstrate effectiveness of bee venom against inflammatory implications like arthritis including rheumatic and osteo-arthritis (Lee et al., 2014). Clinically Bee venom acupuncture therapy was found useful in the treatment of frozen shoulder (Koh et al., 2013).

Efficiency of bee venom in treatment of several degenerative diseases of nervous system like Alzheimer, Multiple Sclerosis (MS), Parkinson has been recorded (Wesselius et al., 2005, Doo et al., 2010). Apamin, a peptide from bee venom is documented to exert neuro-protective effects suggesting usefulness in treatment of Parkinson's disease (Doo et al., 2010).

Lasioglossin II, another peptide from bee venom possesses cytotoxic effect against several cancer cells *in vitro*.(Bandyopadhyay et al., 2013). Application of bee venom against several kinds of cancer cells including lung, renal, prostate, lever, leukaemia and osteosarcoma (Orsolic, 2012).

Bee venom acupuncture therapy proved clinically useful against the chronical back pain (Shin et al., 2012). Further clinical efficacy of bee venom in immunotherapy has been recorded in beekeepers (Münstedt et al., 2010).

Apitherapists or healthcare providers practicing bee venom therapy follow defined treatment protocols. The therapy begins with allergy test by administering minimal dose of bee venom intradermally. In case of no allergic response, its use is continued with one or two injections. Median lethal dose (LD50) of bee venom for human is documented to be 2.8 mg/kg of body weight (Ali, 2012). Bee venom is available as pure liquid venom, whole bee extract or an injectable solution.

1.1.3F. BEES WAX:

Beeswax is a creamy substance produced by bees in their wax glands. Bees utilize this wax for building of comb which forms the assembly of their nest (Bogdanov, S. 2009). Different species of bees produce wax of variable physical and chemical properties. Asian species (*Apis florae*, *Apis cerana* and *Apis dorsata*) produce "Ghedda wax" which is less acidic than that produced by *Apis mellifera*. The bumblebees produce wax different from that produced by honeybees (Krell, 1996). Young worker honeybees secret wax in liquid form from their wax glands. The liquid wax hardens immediately when comes in contact with air and forms small flakes. The bees chew these flakes in their mandibles (jaws) and add salivary secretions to it. This wax is then applied for sealing of honey cells and comb construction. Newly generated wax is white and clear however turns pale yellow upon manipulation by bees (Mutsaers et al., 2005). Beeswax has melting point in the range of 10-20˚C, bears typical odor of bees, propolis, honey, with pleasant taste. The beeswax crystallizes upon storage for about 3-4 months with increased stiffness and elasticity. It is practically insoluble in water, soluble in several organic solvents like acetone, benzene, chloroform, ether, toluene, tetrachlormethane, xylol.

Chemically, beeswax is complex material comprising more than 300 different constituents (Tulloch, 1980). Esters of fatty acids and alcohols comprise the major components of beeswax along with traces of hydrocarbons and aroma compounds. Table 1.6 displays composition documented for beeswax.

Table 1.6. Composition of beeswax:

Component	Quantity (% w/w)
Monoesters	35
Diesters	14
Triesters	3
Hydroxy monoesters	4
Hydroxy polyesters	8
Acid esters	1
Acid polyesters	2
Hydrocarbons	14

Free acids	12
Alcohols	1

[Source: (Tulloch, 1980)]

Several pharmacopoeias use the parameter "ratio of ester values to acids" to determine the quality of beeswax. Heating of the beeswax can affect this ratio which is compared with that of the pure beeswax (Mutsaers et al., 2005).

Literature confers exploration of beeswax mainly for cosmetic and non-medicinal uses (Bogdanov, 2009, Bradbear, 2009). Applications of beeswax in development of cosmetic and skin care products are increasing. It is documented that cosmetics and pharmaceutical industry capture about 40% and 30% (respectively) of the worldwide trade in beeswax (Bradbear, 2009).It is an approved glazing agent (E901) used in the preparation of lip gloss, balms, moisturizers, hand creams, eye shadow and eye liners.

Other applications of bees wax:

- Food additive and used in coating over cheese. It exhibits glazing properties to food materials which also prevents water loss in fruits.
- Several tablet coatings and soft gelatin capsules are made from beeswax.
- It is preferred ingredient in natural and medicated chewing gums.
- On account of its lubricating and emulsifying properties, beeswax is important base of suppositories.

Other uses documented for beeswax include:

- Grafting of waxes
- Lubricants in industrial applications.
- Used in polishes of cars, furniture, shoes and other leather products.
- Used as sealant to bottles or containers
- Used in confectionery coatings.

1.1.4. REGULATORY CATEGORIZATION AND GLOBAL MARKET SCENARIO:

The regulatory authorities worldwide including US Food and Drug Administration (US-FDA), European Medicines Agency (EMA) have categorized the apiproducts 'honey' and 'bees wax' as food or food ingredient. US-FDA's Center for food safety and applied nutrition, issued a draft guidance for industry on proper labeling of honey and honey products (2014) which classifies honey under food and food products. Further according to US-FDA Code of Federal Regulation Title 21, vol 3; 2014 (21CFR184.1973) the beeswax has been categorized as food for human consumption with labeling of Generally Regarded As Safe (GRAS). The allied products like bee pollen, propolis and royal jelly have been considered as dietary supplements by the regulatory authorities worldwide including International Honey Commission (IHC). The US-FDA categorizes bee pollen and royal jelly under the class Y of "Unconventional Dietary Specialty products" (viz. products for humans and animals which are natural supplement sources from plants and animals). Few of the scientific reports also have described bee pollen, royal jelly as a functional food or food additives (Campos et al., 2010).

There exists a scarcity of market information about allied apiproducts like propolis, bee pollen, royal jelly as honey attracts commercial business attention globally. Worldwide the honey market is considered as stable still evolving one. According to European Commission DG Agriculture and Rural Development's report (2013), global honey production in the year 2010 was estimated 1.5 million tones (MT), reaching to 1.9 MT till 2015. Asia and Europe are reported to be the major global producers of honey contributing 43% and 23% respectively to the global production. China stands to be the largest producer of honey with a size of 4,00,000 tons, followed by Turkey and USA (80,000 tons each), Ukraine (70,000 tons), Argentina (60,000 tons), Mexico and Ethiopia (55,000 tons each), Russia and Iran (50 000 tons each). Europe, China and USA are the major honey consumers contributing 20-25%, 15% and 10%

respectively of the global consumption (European Commission Report No 30-CE-0219319/00-20; 2013).

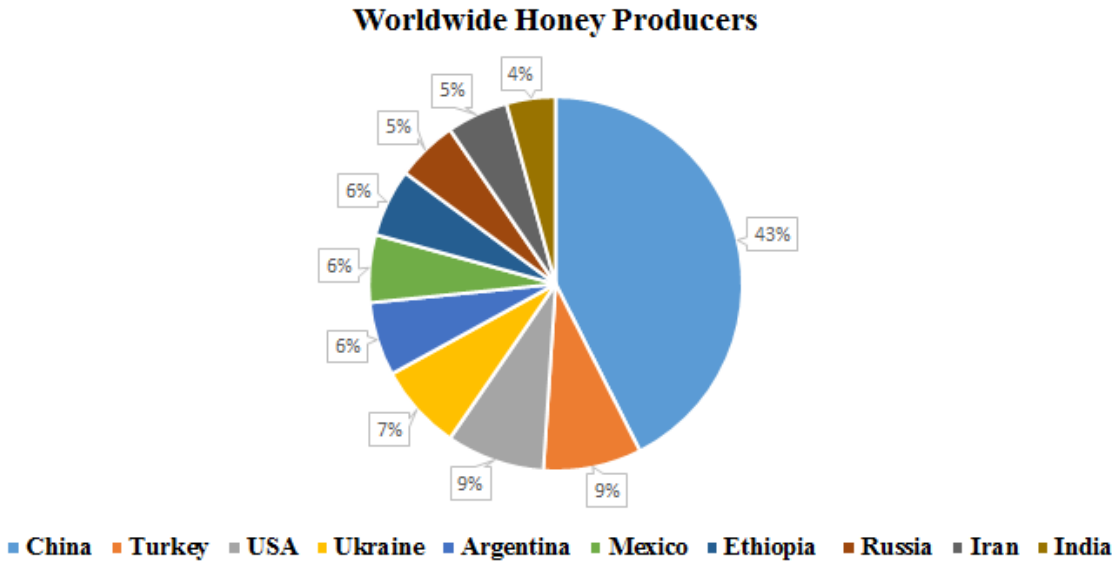


Figure 1.3. Major honey producer countries and their volume contributions worldwide

The United States Department of Agriculture's (USDA) National Agriculture Statistics service reports production of 149 million pounds of honey in 2013 with a market size of $ 315 million. Owing to the higher consumption rates, European countries made around 38.2 % of global honey import. Major honey importer European countries include Germany, United Kingdom, France and Spain. It was followed by North America making 30.1% of global honey import. China on account of largest honey production, leads world honey exports and has been a biggest supplier of honey worldwide. There exists lacuna of official data revealing the exact market size details for other apiproducts such as propolis, bee pollen, royal jelly as honey forms most popular and preferred apiproduct worldwide.

1.1.5. INDIAN PERSPECTIVE:

India ranked 10th position in the list of worldwide honey producers with a size of 40,000 tons (European Commission Report No 30-CE-0219319/00-20; 2013). According to official web portal of Agricultural and processed food products Export Development Authority (APEDA), Government of India, the country exported 28,378.42 MT of honey to the world amounting of Rs. 444.98 crore in 2013-14. Major export regions include US, Saudi Arabia, United Arab Emirates, Bangladesh, Morocco and Canada.

India is known to be a 'land of honey' (Gandhi G.P. 2003). Apiculture has a long history in India. Major regions for apicultural activities in India include Punjab, Jammu-Kashmir, Himachal Pradesh, Uttar Pradesh, Haryana, Bihar and West Bengal (Thomas et al., 2002, More et al., 2010, Ketkar et al., 2014). The forests, farms around sub Himalayan tracts, cultivated vegetation in Madhya Pradesh, Rajasthan, Eastern Ghats in Andhra Pradesh and Maharashtra are known to be the major regions for honey collection (Dalio, 2012). These regions possess rich flora of nectariferous and polliniferous plant species, which are prime requirement of apiculture so as to produce quality pollens and nectar, the raw materials for beekeeping industry. There are species of true honey bees *Apis cerena, Apis dorsata, Apis melliferra* and three species of the stingless bees. Collectively these present a broad variety of bee fauna which can be explored for development and expansion of honey industry in India. On account of these natural resources, beekeeping is being explored as a subsidiary occupation by farmers due to its beneficial returns at low cost investment (Karan et al., 2013).

Despite of availability of abundant natural resources and infrastructure for bee keeping industry, India has not explored its potential in the field of apiproducts to full extent as evident from the available figures for honey market. Further except for honey, no official data is available to trace the market share of allied bee products such as propolis, bee pollen, royal jelly from India. Apart from honey, minimal commercial

exploration of allied apiproducts is observed in India. The deprived success so far can be attributed to limited research and development in the field of apiproducts in India. Along with honey, India can also become an important source of allied apiproducts such as propolis, bee pollen, royal jelly, bee venom, etc.

1.1.6. WAY AHEAD:

With the increasing global as well as domestic consumer demand each year, adoption of advanced apiculture practices (modern hives, good nectar forage areas, production of healthy bees, colony disease management) and development of value added apiproducts, can help to tap the unexplored potential of Indian apiculture to fullest extent.

1.2. GENESIS OF WORK: Development of Value Added Apiceuticals:

- Apart from honey, the bee pollen, propolis, royal jelly and bee venom are important apiproducts which possess extraordinary medicinal values. These are known to be rich source of several active chemical moieties exhibiting array of pharmacological activities. Standardization of such apiproducts in terms of active constituents will contribute to describe the quality of product or finished dosage form produced thereby.
- Exploration of active principles from apiproducts for nutritional and therapeutic applications can contribute to value addition of apiproducts.
- Despite of diverse therapeutic potential of apiproducts, the poor solubility (bee pollen, propolis); waxy or resinous nature (propolis, beeswax); sticky consistency (honey, propolis) and physical instability (royal jelly) impart hurdles in their processing and formulation development. Further large amount of scientific evidences dictate poor digestibility and bioavailability of apiproducts such as bee pollen and propolis. (Metzner 1978, Roulston 2000, Campos 2010). Some of the apiproducts like bee pollen or royal jelly can be consumed in the state in which they are produced by bees, while others like propolis cannot be consumed in raw state. Potentiation of nutritional and therapeutic efficiency of all these products necessitates effective processing of raw apiproducts. Design and development of processing methods by application of pharmaceutical aids will convert raw apiproducts into "Value Added Apiceuticals" with enhanced nutritional and therapeutic efficiency.

In light of these progressions, current work was undertaken as an attempt to investigate the applicability of pharmaceutically acceptable excipients for processing of apiproducts bee pollen and propolis converting them into potentiated or fortified, value added apiceuticals (VAA) with improved therapeutic efficiency. Further attempts were made to develop mechanistic understanding in various aspects of innovative extraction processes used for these apiproducts. We have also initiated fundamental studies on

active moieties originating from propolis, in view of modulating its physicochemical properties with special emphasis on improvement in aqueous solubility.

Accordingly, the studies were designed in two parts:

A) Multicomponent systems:

These include studies on bee pollen and propolis (as a whole or in the form of extracts thereof) in view of nutritional or therapeutic enrichment using pharmaceutically acceptable excipients

B) Single component system:

This include studies on therapeutically active polyphenolic constituent of propolis i.e. Caffeic acid phenethyl ester (CAPE), in view of modulating its physicochemcial properties and improving the aqueous solubility. The study involves application of crystal engineering technique to generate CAPE cocrystals using pharmaceutically acceptable coformers.

1.3. LITERATURE SURVEY:

1.3.1. BEE POLLEN:

Few of the scientific reports disclosing beneficial effects of bee pollen along with the literature documenting morphology and digestibility of bee pollen are discussed below (Table 1.7). Also reports on different methods for processing of bee pollen have been discussed.

Table 1.7. Literature reports for bee pollen

Authors	Findings of research
A) REPORTS ON BENEFICIAL EFFECTS OF BEE POLLEN	
(Campos et al., 1997a), (Campos et al., 1997b), (Campos et al.,	**Antioxidant activity:** Collectively the reports document antioxidant effects and free radicals (DPPH, superoxide, hydroxyl) scavenging activities of bee pollen *in vitro* and *in vivo*. Several of the listed reports demonstrate a close relationship between phenolic

2003), (Almaraz AN et al., 2004), (Leja et al., 2007), (Carpes et al., 2009), (LeBlanc et al., 2009), (Morais et al., 2011), (Feas et al., 2012), (Freire et al., 2012) (Mejías and Montenegro, 2012) (Sousa et al., 2015)	composition of bee pollen and their antioxidant activity. Almaraz et al., 2004 also reported potent lipid peroxidation inhibitory effect of *Amaranthus hybridus* pollen by thiobarbituric acid reactive substances (TBARS) test on hepatic microsomal preparations. LeBlanc et al., 2009 also documented potent antioxidant activity of Mimosa bee pollen in FRAP (ferric reducing-antioxidant power) assay along with DPPH scavenging activity. Feas et al., 2012 also reported lipid peroxidation inhibitory effect of methanolic extract of bee pollen analyzed by β-Carotene bleaching (BCB) assay. Freire et al., 2012 documented antioxidant activities of Brazilian bee pollen based on DPPH assay, Fe_2^+ ion chelating activity assay, and 2,2-azinobis 3-ethylbenzothiozoline-6-sulfonic acid (ABTS) assay in correlation with total phenolic content. Sousa et al., 2015 reported protective role of flavonoid rich fraction of *Echium plantagineum* L. bee pollen against tert-butyl hydroperoxide (t-BHP) induced oxidative stress in Caco-2 cells.
(Juzwiak et al., 1989), (Polanski et al., 1997)	**Hypolipidemic activity** Juzwiak et al., 1989 demonstrated hypolipidemic activity of bee pollen by reducing total lipids and triacylglycerols content of plasma in rabbits. (preclinical investigation) Polanski et al., 1998 reported effective role of bee pollen in hyperlipidemia and atherosclerosis patients by lowering lipids and cholesterol level from 20 to 30% and reduced clumping of platelets for 30%. (clinical investigation)

(Maruyama et al., 2010), (Choi, 2007) (Pascoal et al., 2014)	**Anti-inflammatory activity** Maruyama et al., 2010 reported anti-inflammatory activity of ethanolic extract of *Cistus* sp. bee pollen against carrageenan-induced paw edema in rats. Further it documented the mechanism to be inhibition of NO production and cycloxygenase (COX-2) inhibitory activity. Choi et al., 2007 investigated antinociceptive activity of pine pollen extract against acetic acid induced abdominal constriction, formalin induced licking test in mice. Further it reported anti-inflammatory effect of the extract against arachidonic acid induced ear edema model. Pascoal et al., 2014 reported antioxidant, antimicrobial and anti-inflammatory effects of bee pollen. The anti-inflammatory effect was assessed in terms of inhibitory effect -by hyaluronidase assay.
(Yasumoto et al., 1995)	Clinical efficacy of bee pollen for their anti-prostatic effect in humans was established.
(Wojcicki et al., 1989), (Yıldız et al., 2013)	**Hepatoprotective and detoxifying activity:** Wojcicki et al., 1985 demonstrated protective effect of bee pollen extract against allyl alcohol induced liver damage in rats. Yıldız et al., 2013 reported hepatoprotective effect and detoxifying action of chestnut bee pollen in rats against carbon tetrachloride induced liver damage.
(Medeiros et al., 2008), (Moita et al., 2014), (Geyman, 1993), (Greenberger and	**Anti-allergic effect:** Medeiros et al., 2008 demonstrated anti-allergic effect of bee pollen in ovalbumin sensitized mice. Recently Moita et al., 2014 reported anti-allergic role of *Echium plantagineum* L. bee pollen against β-hexosaminidase

Flais, 2001)	release in rat basophilic leukemic cells (RBL-2H3). (Though anti allergic activity of bee pollen are documented, few researchers like Geyman, 1993; Greenberger and Flais, 2001 have also documented non-life-threatening anaphylactic reactions associated with bee pollen intake).
(Carpes et al., 2007), (Tichy and Novak, 2000),	**Antimicrobial activity** Collectively these reports document antibacterial activity of bee pollen against *Staphylococcus aureus, S. epidermidis, Streptococcus viridans, Pseudomonas aeruginosa, Bacillus subtilis*, Klebsiella sp. Further effective role of Turkish bee pollen against different pathogens for plants viz. *Agrobacterium vitis, A. tumefaciens, P. syringae pv. phaseolicola, P. savastanoi pv. Savastanoi,P. syringae pv. syringae, Ralstonia solanacearum, Pseudomonas corrugata, Xaxonopodis pv. Vesicatoria and Xanthomonas campestris pv. campestris*was reported.
(Wu and Lou, 2007), (Aliyazicioglu et al., 2005), (Izuta et al., 2009)	**Anticancer potential** Wu and Lou 2007 reported anticancer activity of Brassica bee pollen extract which induced apoptosis of human prostate cancer PC-3 cells. Aliyazicioglu et al., 2005 demonstrated inhibition in respiratory burst of K-562 cancer cells upon treatment with bee pollen extract. Izuta et al., 2009 showed that bee pollen extract inhibit proliferation of human umbilical vein endothelial cells.
(Yamaguchi et al., 2006), (Yamaguchi et al., 2007),	**Anabolic activity** Explored anabolic effects of *Cistus ladaniferus* bee pollen extract in femoral tissues of rats bone. In another study the same group reported anabolic effects the pollen extract in

	Osteoblastic MC3T3-E1 cells *in vitro.*
(Turner et al., 2006), (El Asely et al., 2014), (Salles et al., 2014)	**Nutritional effects** Turner et al., 2006 demonstrated that bee pollen supplementation (Dynamic Trio 50/50) improves feed intake into training horses meeting their increasing nutrient demands El Asely et al., 2014 demonstrated that incorporation of bee pollen in diet (2.5%) of *Nile tilapia* fish (*Oreochromis niloticus*) improves growth and immunity of the fish. Further it offered better protection against challenge by *Aeromonas hydrophila*. Salles et al., 2014 reported that administration of diets containing fresh monofloral bee pollen improved muscle protein and energy metabolism in old, malnourished Wistar rats.
(Slijepcevic et al., 1979)	Slijepcevic et al., 1979 reported that supplement of bee pollen for 6 months increased reproduction rates in mice
(Wojcicki et al., 1991)	Wojcicki et al., 1991 reported bee pollen to boost mental capacity and stimulating the nervous system affected by overstress conditions. The study suggests usefulness of bee pollen in the treatment of physical or mental tiredness, apathy and asthenia
(Maughan and Evans, 1982), (Bogdanov, 2014)	**Use in sports people:** Maughan and Evans, 1982 reported a study of controlled experiments in swimmers receiving bee pollen supplementation for 27 days wherein no significant improvement in performance of swimmers was recorded, however the bee pollen treated groups demonstrated decline in number of missed training days due to upper respiratory tract infections when compared with placebo group.

	Bogdanov, 2014 cited that per day intake of 10 g bee pollen was reported to improve athletic performances by national team of Lithuania along with increase in blood haemoglobin values.
B) REPORTS ON MORPHOLOGY, DIGESTIBILITY AND PROCESSING OF BEE POLLEN	
(Wiermann and Gubatz, 1992), (Roulston and Cane, 2000), (Pacini and Hesse, 2005), (Kovacik et al., 2009)	Collectively these reports described about bee pollen structure which comprises cytoplasm enclosed within a multilayered tough pollen coat comprising of exine and intine. Roulston and Cane, 2000 stated that the ridged matrix composition of coat makes the pollen rigid thereby resists its decay and digestion. These impart low digestibility to bee pollen limiting their usefulness for consumption by humans, on account of highly impervious cell wall structure.
(Brooks and Shaw, 1978), (Burczyk and Dworzanski, 1988), (Espelie et al., 1989), (Wittborn et al., 1996), (Diego-Taboada et al., 2014)	These reports documented composition of pollen wall which comprises of inner layer 'intine' made of cellulose and pectin, the outer layer 'exine' consisting mainly of sporopollenin. It is reported to be the most resistant substance against physical or chemical treatments including alkali and acids. Exact chemical composition of sporopollenin is yet imprecisely known. Brooks and Shaw 1978 reported chemical composition of sporopollenin to be an oxidative polymer of carotenoids. Further it also comprises polyunsaturated fatty acids in its composition. Diego-Taboada et al., 2014 cited that the elemental analysis documents it to be nitrogen free.
(Loewus et al., 1985),	Loewus et al., 1985 reported dissolution of sporopollenin component of pollen wall in 4-Methylmorpholine N-oxide

(Sreedevi et al., 1990)	monohydrate. Sreedevi et al., 1990 studied the solubility differences in sporopollenin and pollinial wall of pollen, documented insoluble nature of sporopollenin (except in hot 2-aminoethanol).
(Franchi et al., 1997), (Campos et al., 2010)	Franchi et al., 1997 studied digestibility of poppy and hazelnut pollen grains in human-like *in vitro* digestion conditions with pancreatic enzymes. Results confirmed insufficient digestion of both pollen grains even after 24 h treatment. This was ascribed to inability of enzymes to penetrate the intine of pollen grains. Based on the findings, Campos et al, 2010 cited a hypothesis that cracking of pollen would improve the digestibility and bioavailability of pollen nutrients.
(Du Lianxiang, 2004)	Reported solid state aerobic fermentation of bee pollen using *Lactobacillus acidophilus* and *Bacillus natto*, at 1:1 ratio for 3 days at 30℃. The fermentation process was reported to potentiate the bee pollen in terms of armor, taste, natural functions with probiotic characters.
(He et al., 2006), (Ruan et al., 2008)	He et al., 2006 reported application of four different methods as wall-breaking treatments to corn pollen. The methods include: a) temperature difference method; b) ultrasonic method; c) enzyme method and d) synthesis method. The wall-breaking ratios of the enzyme method and the synthesis method were documented to be significantly higher than temperature difference method and ultrasonic method. Ruan et al., 2008 applied six different methods for breaking the rape bee pollen wall and monitored the extraction of flavonoids. The methods include ultrasonication; temperature

	difference, enzymolysis; temperature difference-ultrasonic method, temperature difference-enzymolysis method and integrated method of the three single methods. Results of study demonstrated that application of integrated method increased flavonoids extraction 31.5% higher than that of untreated pollen (2.2447 mg/g). Further the application of integrated method demonstrated 86.6% increase in wall breaking rates leading to improve the extraction yield of flavonoids as analyzed by HPLC.
(Fang et al., 2008)	Reported method for isolation of de-exined pollen (*Pinus bungeana* Zucc. Ex Endl. and *Picea wilsonii* Mast) by incubating with sucrose solution (12–15%), boric acid (0.01%), calcium chloride as base (0.01%) and enzymes cellulase R-10 (0.5–1.5%) and macerozyme R-10. (0.8%) 30 min at 24 ˚C.
(Xu et al., 2009a) (Wang et al., 2009)	Xu et al., 2009a studied application of supercritical carbon dioxide technology for breaking the wall of rape bee pollen followed by consecutive extraction of lyzed pollen oil. The pollen treated with supercritical CO_2 at 45 MPa / 10 min followed by depressurizing achieved improved extraction of 5.98 g oil /100 g of dry pollen. Similarly, Wang et al., 2009 reported application of SFE-CO_2 for extraction of lipids from rape bee pollen and concluded that SFE-CO_2 is more selective method than conventional petroleum ether extraction as analyzed by HPLC.
(Fíla et al., 2011)	Studied impact of different homogenization protocols and protein extraction conditions on proteomic patterns of tobacco pollen. Roche MagNA Lyser Instrument was used for homogenization purpose. The study concludes that the

	proteomic pattern of pollen does not rely solely on homogenization process, but also depend on the extraction protocol followed in study.
(Tabuchi et al., 2011)	Reported weakening and solubilization of cell wall matrix of maize silk by application of purified β-expansin (group-1 allergen) from maize pollen.
(Rzepecka S et al., 2012)	Documented that pretreatment of bee pollen with pepsin (1%) in distilled water (acidified with conc hydrochloric acid to pH 2), incubated at 37˚C/ 48 hr followed by extraction in hydroalcholic solution enhanced its antioxidant effect by increasing quenching of paramagnetic centers in DPPH activity as analyzed by electron paramagnetic resonance technique.
(Lv et al., 2015)	The study reports application of different methods for extraction of flavonoids from rape bee pollen preparation "Qinghai-Tibetan Plateau". The methods include a) Soxhlet extraction, b) microwave-assisted extraction, c) cold soaked extraction and d) heat reflux extraction. Further an HPLC method for estimation of flavonoid aglycones from pollen was developed. Results of study document highest flavonoids extraction yield by microwave assisted extraction method.

Patent Literature: Table 1.7.1 lists few of the patents related to bee pollen claiming its compositions, method of use are as follows:

Table 1.7.1. Patent literature for bee pollen

Patents/ application No.	Description
US 8,679,522	Discloses a chewing gum with a liquid-fill composition containing bee pollen

US 8,197,867	Discloses a three layered dietary supplement composition containing bee pollen.
US 7,879,343	Discloses method of treating burn using bee pollen composition.
US 7,410,654	Discloses pollen manufacturing method using electrolysis reducing water.
US 7,182,965	Compositions containing sporopollenin and use thereof.
US 5,744,187	Discloses nutritional powder composition containing energy boosting natural products (including bee pollen), readily soluble in a fluid for ingestion by humans.
US 6,579,543	Discloses composition for topical application for skin infections.
US 6,287,567	Discloses method of making herbal drink containing pollen as ingredient.
US 4,426,397	Discloses process for production of concentrate containing bee products
EP 1077725B1	Discloses use of mixture of pollen extract and vegetable oil for preparation of topical or transdermal medicament for dermatological, inflammatory conditions.
EP 1313492B1	Water soluble and/ or fat soluble cytosolic extract of pollen with Royal jelly, Vitamin E for treatment of dysphoria.
CN103704550 A	The invention relates to bee pollen wall breaking dissociation liquid which comprises water, starch, yeas cell wall polysaccharide, glutathione, VE, citric acid, oxalic acid and 1-hydroxyethylidene-1,1-diphosphonic acid.
CN101971939 A	Discloses process for making freeze-dried bee pollen powder by low-temperature high-pressure wall-breaking.

1.3.2. PROPOLIS:

Few of the scientific reports about beneficial effects of propolis along with different methods for processing / extraction are discussed in table 1.8 as follows:

Table 1.8. Literature reports for propolis:

Authors	Findings of research
A) REPORTS ON BENEFICIAL EFFECTS OF PROPOLIS	
(Kumazawa et al., 2004), (Lagouri et al., 2014), (Selamoglu et al., 2015), (Zhang et al., 2015), (Lahouel et al., 2010),	**Antioxidant activity:** Collectively these studies report *in vitro* and *in vivo* antioxidant effects of propolis. Kumazawa et al., 2004 reported antioxidant activity in terms of β-carotene bleaching assay and free radical (DPPH) scavenging assay for propolis from different geographic origins (Austria, Argentina, Bulgaria, Brazil, China, Chile, New Zealand, Hungary, Thailand, South Africa, Uruguay, Ukraine, Uzbekistan and USA). Propolis from Australia, Argentina, New Zealand, Hungary and China demonstrated relatively higher antioxidant effects which were in correlation with the polyphenol and flavonoid contents. Lagouri et al., 2014 analyzed major polyphenolics extracted from Greek propolis using polar solvents Further antioxidant effect of the extracts was analyzed in terms of free radical (DPPH) scavenging assay and ferric reducing antioxidant power (FRAP) assay. Selamoglu, et al., 2015 reported antioxidant effects of propolis in terms of inhibitory effect on catalase activity, nitric oxide and malondialdehyde (MDA) levels in liver tissues triggered with Nω-Nitro-L-arginine methyl ester (L-NAME) Zhang et al., 2015 reported antioxidant effect of propolis on expression of antioxidant genes (TrxR1, HO-1, GCLC and

	GCLM) in RAW264.7 cells. Lahouel et al., 2010 documented effectiveness of propolis polyphenols in alleviating renal oxidative stress induced by doxorubicin.
(Kujumgiev et al., 1993), (Marcucci, 1995), (Kujumgiev et al., 1999), (Salomao et al., 2004), (Fokt et al., 2010), (Bonvehí and Gutiérrez, 2012) (Boisard et al., 2015),	**Antibacterial, antifungal, antipathogenic activities:** The studies collectively confer a concentration dependent static or cidal activity of propolis against broad spectrum of Gram positive bacteria [Bacillus spp. (*B. cereus, B. subtilis*), Staphylococcus spp. *(S. aureus, S. auricularis, S. epidermidis, S. capitis, S. hominis, S. haemolyticus, S. mutans, S. warnerii*), *Micrococcus luteus,* Enterococcus spp. (*E. faecalis*), *Rhodococcus equi, Nocardia asteroids,* Streptococcus spp. (*S. faecalis, S. Pneumonia, S. cricetus, S. pyogenes, S. sobrinus, S. mutans, S. β-haemolyticus, S. viridians*), *Peptostreptococcus micros*, *Actinomyces naeslundii*, *Lactobacillus acidophilus*] as well as Gram negative bacteria *Aeromonas hydrophila, Escherichia coli, Pseudomonas aeruginosa,* Salmonella sp. *(S. typhi, S. typhimurium, S. enteritidis,), Actinobacillus, Klebsiella pneumonia, Brucella abortus.* Studies also report activity of propolis against pathogens like Corynebacteriumsp. *(C. pseudotuberculosis),Proteus vulgaris, Proteus mirabilis, Shigella dysenteriae, actinomycetemcomitans, Porphyromonas gingivalis, Porphyromonas anaerobius, Capnocytophaga gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Prevotella oralis, Prevotella melaninogenica, Veillonella parvula* Boisard et al 2015 report antifungal properties of French propolis against pathogenic strains *Aspergillus fumigatus, Candida albicans,* and *C. glabrata* (Boisard et al., 2015)

(Torres et al., 1990) (Salomao et al., 2011)	**Antiprotozoan activity** Collectively these reports document antiprotozoan activity of propolis against *Giardia lamblia, Toxoplasma gondii*, *Trichomonas vaginalis*, *Leishmania donovani*, etc, indicating its use in richomoniasis, giardiasis, leishmaniasis, toxoplasmosis, malaria.
(Boyanova et al., 2003) (Boyanova et al., 2005)	**Anti-*Helicobacter pylori* activity and hepatoprotective effect** Boyanova et al., 2003; 2005 demonstrated potent and dose-dependent activity of Bulgarian propolis against *H. pylori strains*. (*In vitro*) These studies document hepatoprotective effects of propolis.
(Bankova et al., 2014a) (Kai et al., 2014)	**Antiviral activity** Bankova et al., 2014a reported virucial effect of Propolis Extract ACF® against herpes simplex (type 1 and 2) viruses in MDBK cell culture. Kai et al., 2014 reported anti-influenza virus activity of flavonoids from Propolis. (both *In vitro* and *in vivo*)
(Kubina et al., 2015) (LS, 2014) (Xuan et al., 2014) (Choudhari et al., 2013) (Vit et al., 2015)	**Anticancer activity** Stockpile of reports dictate antitumor effects including cytotoxic activity of propolis in different *in vitro* cell line and *in vivo* models. Kubina et al., 2015 recently documented antiproliferative, pro-apoptotic activity of ethanolic extract of Polish propolis on Me 45 malignant melanoma and HCT 116 colon cancer. Kumar LS et al., 2014 reported a review on use of propolis in oral cancer therapy and dentistry. Xuan et al., 2014 demonstrated antitumor effects of Chinese propolis in MCF-7 and MDA-MB-231 cells (in human breast cancer).

	Choudhari et al., 2013 reported cytotoxic activity and apoptotic effect of ethanolic extract of Indian propolis different cell lines, HT-29 (human colon adenocarcinoma), Caco-2 (human epithelial colorectal adenocarcinoma), MCF-7 (human breast cancer), and B16F1 (murine melanoma). Vit et al., 2015 summarized a review on anticancer activities of propolis and CAPE. The review documents role propolis and its constituents to induce apoptosis pathways in cancer cells.
(Sforcin, 2007) (M de Figueiredo et al., 2014)	**Immunomodulatory activity** Propolis is reported to exhibit immunomodulatory and antimutagenic properties. Sforcin, 2007 and Figueiredo et al., 2014 reviewed immunomodulatory potential of propolis and documented beneficial effects of propolis on immune system.
(Araujo et al., 2012) (Naik et al., 2013a) (Anauate-Netto et al., 2014)	**Anti-inflammatory and analgesic effects** Arjuno et al., 2012 reviewed different mechanisms of action behind the anti-inflammatory effects of propolis. Inhibition of COX, prostaglandin and nitric oxide synthesis, free radical scavenging action, decreasing the inflammatory cytokines and immunosuppression are the main mechanisms documented. Naik et al., 2013a demonstrated anti-inflammatory activity of Indian propolis against 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear oedema and carrageenan induced rat paw oedema model. Anauate-Netto et al., 2014 reported clinical effectiveness of propolis mouthrinse in reducing the Papillary Bleeding Score (PBS) on mesio-buccal surfaces confirming use in gingivitis.
(Daleprane and Abdalla, 2013) (Tang et al., 2015)	**Cardioprotective, antidiabetic and neuroprotectiveeffects** Daleprane and Abdalla, 2013 reported a review on cardioprotective effects of propolis which document effects of

(Farooqui, 2012)	propolis in regulating the glucose and lipoprotein metabolism; alteration of gene expression; reduction in inflammatory cytokines, reaction in activity of scavenger receptors, alleviating oxidative stress; improving the endothelial function; and inhibiting the platelet aggregation. Tang et al., 2015 demonstrated hypolipidemic and hypoglycemic effects of propolis in Streptozotocin induced Type 2 diabetic rats administered with high fat diet. Farooqui, 2012 documented use of propolis in age induced neuro-inflammation associated with age-related diseases
B) REPORTS ON PROCESSING / EXTRACTION OF PROPOLIS:	
(Krell, 1996) (Mutsaers et al., 2005)	Krell, 1996 and Mutsaers et al., 2005 collectively document procedures to be followed in processing of raw propolis obtained from bee hives. The propolis needs to be cleaned for removing excessive wax, lead and other extraneous matter. The cleaned propolis should be subjected to freezing to make it brittle and non-sticky which enables grinding of propolis for extraction purpose.
(Park and Ikegaki, 1998); (Bankova et al., 2002b) (Cunha et al., 2004) (Liu and Wang, 2004) (Marquele et al., 2005)	Park and kegaki, 1998; Bankova et al., 2002 report poor aqueous solubility of propolis and its active constituents. Cunha et al., 2004 studied factors which influence the extraction phenolics from Brazilian propolis. The study compared maceration and soxhlet methods of extraction. Propolis was subjected to maceration for 7, 10, 20, 30 days with occasional shaking at room temperature. Different concentrations 30, 50, 70 and 100% of absolute alcohol were used for extraction in comparison with water. In another protocol maceration was continued for 30 days with solvent renewal after every 7 days. For soxhlet extraction, the propolis

	was subjected to Soxhlet extractor for 24 at 60 °C. Extracts obtained through both Soxhlet and maceration procedures subjected to freezing to induce the crystallization of dissolved waxes. Results of the study confirmed that maceration achieved highest extraction yield using 70% (v/v) ethanol. Increasing yield of extracts were observed with maceration from 10 to 30 days. Liu and Wang, 2004 reported extraction of flavonoids from propolis using ultrasound method (power 750W for time 15mins) using ethanol as solvent. Marquele et al., 2005 document extraction of Brazilian propolis using polyethylene glycol. The glycolic extract of propolis demonstrated comparative polyphenols, flavonoids content and antioxidant activities with that of ethanolic extract of propolis.
(Cvek et al., 2007) (Trusheva et al., 2007) (Coneac et al., 2008) (Margeretha et al., 2012) (Papotti et al., 2012) (Khacha A et al., 2013)	These reports demonstrate improved extraction of propolis polyphenolics and flavonoids in different organic solvents like ethanol, methanol, ethyl acetate, acetone, n-hexane, chloroform. (Cvek et al., 2007)reported optimization of process for extraction of flavonoids from Crotian propolis. Different conditions for extraction (time, temperature and concentration of extraction solvent- ethanol: water) were optimized to achieve highest extraction. Trusheva et al., 2007 studied comparative extraction of active components from propolis by traditional maceration, ultrasound extraction (UE) and microwave assisted extraction (MAE). The study concludes that UE and MAE methods provide higher extraction yield in short timeframes when compared to traditional maceration method. Coneac et al., 2008 studied ‘cold’ and ‘hot’ extraction of

	flavonoids from propolis in different concentrations of hydroalcoholic solvents at room temperature and at higher temperature 65 ˚C. The extracts produced were analyzed by HPLC. The results of study indicated that 'hot' extraction (65 ˚C) in hydroalcoholic solvent (60 to 96%) achieved higher extraction of flavonoids in terms of rutin, caffeic acid, quercetin, apigenin, kaempferol and chrysin. Margeretha et al., 2012 documented that water can dissolve only a small portion of propolis constituents, (about 10% of its weight) due to poor aqueous solubility of the flavonoids, phenolics and waxes. The study compared three methods of extraction: maceration, reflux, and microwave-assisted extraction. Papotti et al., 2012 determined antioxidant activity and composition in terms of balsams, waxes, resins, flavonoids and total phenolics for Italian poplar propolis. Different solvents ethanol, acetone, and chloroform were used for extraction. Khacha A et al., 2013 reported antioxidant activity and cytotoxicity against HeLa cancer cell lines for propolis extracts produced by two methods viz. maceration and sonication using ethanol solvent (70%). The results demonstrated higher propolis extraction yield with maceration (18.1%) than obtained by sonication (15.7%).
(Jang et al., 2009) (Moret et al., 2010) (Pellati et al., 2013)	Jang et al., 2009 reported optimization of microwave-assisted extraction method for propolis using ethanol as solvent. The method uses combination of microwave with a shearing operation aimed to improve the rate of extraction. Moret et al., 2010 demonstrated extraction of polycyclic aromatic hydrocarbons (PAHs) from Italian propolis using

	microwave assisted saponification followed by extraction. Pellati et al., 2013 reported closed vessel microwave assisted extraction of polyphenols from propolis followed by its HPLC analysis using fused-core technology.
(Wang et al., 2004), (Biscaia and Ferreira, 2009), (Erdogan et al., 2011), (Lombardo A et al., 2012), (Petelinc et al., 2013), Wang, Z. et al 2014	**Sophisticated techniques:** Wang et al., 2004 reported supercritical fluid extractive fractionation of propolis. Further Biscaia, D. 2009 reported comparative study on low pressure methods (maceration, soxhlet extraction) and supercritical fluid extraction of propolis. Results of the study demonstrated highest extraction yield (73 ± 2%, w/w) by chloroform soxhlet extraction over the SFE (24.8 ± 0.9%) Erdogan et al., 2011 reported pressurized liquid extraction (PLE) method for Anatolia propolis and determined its radical scavenging activities. Parameters pressure (500-2000 psi), temperature (20-80 ˚C), type of solvent, cell size (11, 22 ml) and extraction time (15-120 min) were optimized. Different solvents employed include ethanol: ethyl acetate: water: HCl (70:15:10:5), methanol: HCl (95:5), ethanol: water: HCl (70:25:5), methanol: water: HCl (70:25:5), ethanol: acetone: water: HCl (70:15:10:5), water: HCl 95:5; (v/v). Highest phenolics yields in solvent system methanol:water:HCl (70:25:5 v/v). In PLE, solvents maintain liquid state well above their boiling points, which improve phenolics solubility and desorption kinetics from herbal matrices. Lombardo A et al., 2012 developed a dispersive solid phase extraction method 'QuEChERS' (Quick, Easy, Cheap, Effective, Rugged, And Safe) technique, for extraction of

	quinolones from honey, royal jelly and propolis. The extracts produced were analyzed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Petelinc et al., 2013 reported application of solid phase extraction (SPE) for cleaning crude propolis extract. Propolis was extracted with ethanol (70%) and subjected SPE for fractionation into 5 different elution fractions (EL) based on polarity (ethanol 30 to 70%). The EL 60 and EL 70 demonstrated highest phenolic contents as analyzed by LC-DAD technique. Further the fractions also demonstrated highest DPPH scavenging activity and decrease in intracellular oxidation in yeast *S. cerevisiae.*

Patent Literature: Few of the patents and applications related to propolis claiming its compositions, uses and extraction methods are provided in table 1.8.1 as follows:

Table 1.8.1. Patent literature for propolis

Patents/ application No.	Description
US 9,028,827	Discloses composition comprising propolis for treatment of hepatitis virus infection
US 9,005,680	Discloses encapsulated oral composition of propolis extract with triclosan and a fluoride ions source. Composition such as mouth rinse, dentifrice, confectionary, or film are covered.
US 8,722,106	Discloses a lip balm composition of propolis
US 8,455,007	Discloses a cosmetic product comprising aqueous, alcoholic or oil extract of propolis obtained by successive extraction method.
US 8,257,747	Discloses a method to treat propolis. The method involves successive extraction of propolis by water, alcohol and oil.

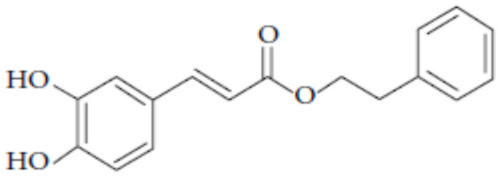
US 7,879,343	Discloses composition of propolis for burn treatment.
US 7,763,282	Discloses a powdered composition of propolis with hydrophilic carrier cyclodextrins
US 7,666,451	Discloses a topical composition of propolis for treatment of dyshidrosis and dry skin disorders
US 7,294,351	Discloses a composition for fractioned propolis wax isolated without use of solvents.
US 6,680,074	Disclose antibacterial, antiviral and antimycotic composition of propolis with essential oils
US 6,190,703	Discloses process for preparing air freshener composition of propolis
US 6,171,605	Discloses self-tanning compositions of propolis extract with DHA
US 6,153,228	Discloses a deodorized propolis extract
US 6,153,227	Discloses a method of producing deodorized propolis extract
US 6,153,226	Discloses antiviral composition of propolis
US 6,106,867	Discloses gelatinous propolis food product
US 5,922,324	Discloses a method for preparing propolis extract with improved water solubility
US 5,692,685	Discloses a process for generating propolis food.
US 5,576,005	Discloses propolis composition for treatment of warts
US 5,561,116	Discloses a method for preparing water dispersible propolis solid composition
US 5,529,779	Discloses novel propolis extract in a water soluble organic solvent
US 5,399,349	Discloses method of treatment for acne using propolis composition
US 4,382,886	Discloses method for extraction of propolis
US 3,960,329	Discloses method for separating and purification of propolis
WO 2011092511	Discloses a method for treatment and use of deodorized and soluble propolis containing composition.

IN 220012	Discloses dried powder composition of propolis with hydrophilic carrier and two co-grinding auxiliary substances (aminoacid and glycyrrhizate)
IN 221711	Discloses process for preparing a novel medicament mixture of propolis with honey, olive oil, camomile, sage, aloe vera, thyme, lavender and/or various oils.
IN 1827/DEL/2009	Discloses a potent antioxidant and hepatoprotective composition of propolis and pocess for preparation thereof

As evident from the literature, propolis exhibits wide array of biological activities, most of which have been ascribed to the polyphenols especially the flavonoids composition of propolis. Caffeic acid phenethyl ester (CAPE) is the most comprehensively studied, therapeutically active polyphenolic constituent of propolis. It has been reported to demonstrate potent anticancer antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, antiviral, neuro-protective effects in several *in viro* cell line and preclinical studies. Following section provides a literature review focusing on different beneficial effects, physicochemical and biopharmaceutical properties documented for CAPE.

1.3.3. CAFFEIC ACID PHENETHYL ESTER (CAPE):

Chemically, CAPE is 2-phenylethyl (2E)-3-(3,4-dihydroxyphenyl) acrylate. Also called as phenethyl caffeate or phenylethyl caffeate. IUPAC name: (E)-3-(3,4-dihydroxyphenyl)-2-propionic acid, 2-phenylethyl 3-(3,4-dihydroxyphenyl)-2-propenoate. It has empirical formula of $C_{17}H_{16}O_4$with molecular weight 284.3 (Murtaza et al., 2014a). It constitutes white crystalline powder freely soluble in organic solvents ethanol, methanol, acetone, DMSO (10mg/ml) while practically insoluble in water. (Tolba et al., 2013).



Caffeic acid phenethyl ester

Few of the references explaining different pharmacological activities, physicochemical and biopharmaceutical properties documented for CAPE are provided in table 1.9 as follows:

Table 1.9. Literature reports for CAPE:

Authors	Findings of research
A) REPORTS ON BENEFICIAL EFFECTS OF CAPE	
(Grunberger et al., 1988), (Liao et al., 2003), (Chen et al., 2004), (Xiang et al., 2006), (Kuo et al., 2006), (Chen et al., 2008), (Ozturk et al., 2012), (Kuo et al., 2013), (Lin et al., 2015)	**Anticancer activity:** Large number of scientific studies document antitumor and cytotoxic activity of CAPE. Grunberger et al., 1988 for the first time reported preferential cytotoxicity of CAPE against on tumor cells. Liao et al., 2003 demonstrated inhibitory effect of CAPE on angiogenesis, metastasis and tumor invasion in BALB/c miceCT26 **colon adenocarcinoma cells**. Chen et al., 2004 reported cytotoxicity of CAPE against **human lung cancer A549 cells** Xiang et al., 2006 investigated effects of CAPE on cell cycle, apoptosis, cell growth and signaling of β-catenin/T-cell factor in **human colon cancer cells.** The results of study demonstrated complete inhibition of cell growth and G1 phase arrest by CAPE with induction of apoptosis in HCT116 and SW480 cells. Further it reduced transcriptional activity of β-catenin/T-cell factor in the cells. Kuo et al., 2006 reported **antitumor activity** of CAPE by

	recording its inhibitory effect on the growth of **glioma cells** (both *in vitro* and *in vivo*) Chen et al., 2008 demonstrated apoptosis inducing effect of CAPE in **human pancreatic cancer cells**. The study documents activation of caspase-3/caspase-7 activity and mitochondrial dysfunction by induced by CAPE. Ozturk et al., 2012 reviewed anticancer mechanism of CAPE against **melanomas, prostate and lung cancers**. Kuo et al., 2013 demonstrated suppression of cell proliferation and survival of **human oral cancer cells** (TW2.6). Recently, Lin et al., 2015 reported effectiveness of CAPE against **prostate cancer** by inducing cell cycle arrest and inhibition of growth in castration-resistant prostate cancer cells.
(Natarajan et al., 1996) (Bharti and Aggarwal, 2002) (Michaluart et al., 1999) (Murtaza et al., 2014b)	**Anti-inflammatory and Immunomodulatory activity:** Natarajan et al.,1996 for the first time demonstrated inhibitory effect of CAPE on NF-kappa B transcription factor (a factor responsible for inflammatory, immune processes) in human histiocytic U937 cell line. The results of study revealed that CAPE blocks activation of NF-kappa B induced by tumor necrosis factor (TNF) in dose dependent manner, conferring inflammatory and immunomodulatory effects. (Bharti and Aggarwal 2002 documents elevation of NF-kappa B (NF-*k*β) transcription factor as one of the underlying cause for promoting tumerogenesis. Thus, CAPE by inhibiting NF-kappa B transcription factor also exerts chemo-preventive role.) Michaluart et al., 1999 demonstrated CAPE induced inhibition of prostaglandin (PG) synthesis in cultured human epithelial cells. Based on review of literature, Murtaza et al., 2014a documents

	mechanism of anti-inflammatory and immunomodulatory effects of CAPE as: a) it induces inhibition of arachidonic acid release from cell membrane thereby inhibits COX-1 and 2 activity; and b) it down regulates activation of gene responsible for COX-2 expression. Further the report also documents that CAPE exerts immunosuppressive effects by inhibiting the interleukin (IL-2) synthesis and IL-2 gene transcription in stimulated T-cells (which are causative agent for inflammation)
(Kishimoto et al., 2005b) (Velazquez et al., 2007) (Ojeda et al., 2008)	**Antimicrobial activity:** Kishimoto et al., 2005 demonstrated *in vitro* antimicrobial activity of CAPE against *Bacillus subtiiis, Staphylococcus aureus*, and *Pseudomonas aeruginosa.* The study also reported antiviral (anti-influenza) and antimutagenic effects of CAPE. Velazquez et al., 2007 demonstrated potent growth-inhibitory activity of CAPE against Gram positive bacteria (*Staphylococcus aureus. Listeria monocytogenes, Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa, Escherichia coli*). Further the study reports free radical scavenging activity of CAPE. Ojeda et al., 2008 reported fungicial activity of CAPE against *Alternaria alternate.*
(Burke et al., 1995), (Shen et al., 2013)	**Antiviral activity:** Burke et al., 1995 reported effectiveness of CAPE as potential anti HIV therapy by demonstrating its antiviral activity against HIV-1 integrase. Shen et al., 2013 reported anti-hepatitis C virus activity of CAPE by demonstrating effectiveness of CAPE against replication of hepatitis C virus in HCV replicon cell line (genotype 1b)
(Tolba et al., 2014)	**Antioxidant activity:**

(Russo et al., 2004), (Wang et al., 2008), (Gocer and Gulin, 2011), (Esrefoglu et al., 2012), (Sahin et al., 2013)	Tolba et al., 2014 reported a review on antioxidant and cytoprotective activities of CAPE against ischemia reperfusion induced injury in several tissues (skeletal muscles, retina, brain, heart, intestine, colon, liver, testis and ovaries). Russo et al., 2002 studied comparative antioxidant properties of propolis extract (with and without CAPE).The antioxidant properties in terms of free radical scavenging effect, xanthine oxidase activity, and anti-lipoperoxidative capacity were investigated. The study concluded that propolis extract (with CAPE) proved more active than propolis extract without CAPE. Further the CAPE alone demonstrated a strong antioxidant activity in terms of DPPH scavenging effect and inhibited xanthine oxidase activity. Wang et al., 2008 revealed cytoprotective potential of CAPE by investigating inhibitory influence on erythrocyte membrane lipid peroxidation, protein fragmentation and cellular DNA strand breakage. The results concluded that CAPE confers potent antigenotoxic and cytoprotective agent. Gocer and Gulcin, 2011 established correlation between structure of CAPE with its antioxidant properties. The study investigated antioxidant effects of CAPE by several methods like total antioxidant activity (thiocyanate method), Free radicals (DPPH, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, superoxide anion, N,N-dimethyl-p-phenylenediamine dihydrochloride) scavenging activities, reducing power and ferrous ions Fe(2+) chelating activities. Further the study also documents 97.9% inhibition of lipid peroxidation of linoleic acid emulsion. Esrefoglu et al., 2012 demonstrated protective role of CAPE

	against aging associated oxidative injury in rat kidneys. The CAPE was documented to induce reduction in malondialdehyde (MDA) levels, elevated catalase (CAT), superoxidae dismutase (SOD), glutathione peroxidase (GPx) activities. Also it restored the glutathione level (GSH) in kidney tissues of old rats. of ganglion cell layer in ratina.
(Kerman et al., 2012)	**Neuroprotective activity** Kerman et al., 2012 reported neuroprotective effects of CAPE on experimental traumatic brain injury (TBI) in rats. CAPE (10 μmol/kg) showed protective effect by decreasing the TBI induced elevated tissue malonaldehyde (MAD) level. Further it significantly increased antioxidant enzyme activities (SOD and GPx) hampered by TBI.
(Cikman et al., 2015)	**Radio-protective activity** Cikman et al., 2015 demonstrated radio-protective effects of CAPE in liver tissues of rats subjected to total head irradiation.
(Yagmurca et al., 2004) (Ozen et al., 2004) (Yagmurca et al., 2004) (Fadillioglu et al., 2004) (Oktem et al., 2006)	**Use in chemotherapy associated toxicities** Yagmurca et al., 2004 demonstrated protective effect of CAPE against doxorubicin induced nephrotoxicity in rats. Ozen et al., 2004 reported protective role of CAPE against nephrotoxicity induced by cisplatin in rats. Fadillioglu et al., 2004 reported protective effect of CAPE against cardiotoxicity induced by doxorubicin. Fadillioglu, et al., 2004 reported protective effect of CAPE against neuronaloxidant injury induced by doxorubicin in brain tissues. Oktem et al., 2006 demonstrated antioxidant effects of CAPE in renal oxidative stress implications associated with Methotrexate treatment.

(Park et al., 2008)	**Anti-allergic activity:** Park et al., 2008 reported anti-allergic effect of CAPE against IgE-mediated, ovalbumin (OVA) induced systemic anaphylaxis in mice
B) REPORTS ON PHYSICOCHEMICAL AND BIOPHARMACEUTICAL PROPERTIES DOCUMENTED FOR CAPE	
(Bankova et al., 1987) (Grunberger et al., 1988)	Bankova et al., 1987 for the first time reported CAPE as a constituent of propolis based on the GC/MS characterization of phenolics. Grunberger et al. 1988 reported method for extraction of CAPE from propolis and demonstrated hydrophobic nature of polyphenol CAPE.
(Bankova, 1990) (Boutagy and Thomas 1974)(Chen et al.,1999) (Son et al., 2001) (Xia and Hu 2005); (Kishimoto et al., 2005a) (Stevenson et al., 2006) (Widjaja et al., 2008)	Stockpile of reports document different methods for synthesis of CAPE. Boutagy and Thomas, 1974; Bankova, 1990 demonstrated synthesis of CAPE based on Witting reaction by using 3,4-Dihydroxy Benzaldehyde as a Substrate Chen et al., 1999 demonstrated synthesis of CAPE by esterification of caffeic acid through acyl chlorides. Son et al., 2001 reported synthesis of CAPE by esterification of caffeic acid with phenethyl alcohol. The study also reported single crystal X-ray diffraction data for benzene solvate of CAPE. Kishimoto et al., 2005 demonstrated enzymatic synthesis of CAPE by transesterification of chlorogenic acid using hydrolase enzyme. Stevenson, 2006 demonstrated *Candida antarctica* lipase catalyzed synthesis of CAPE using caffeic acid and phenethyl alcohol.

	Widjaja et al., 2008 reported enzymatic synthesis of CAPE form caffeic acid and phenethyl alcohol using Novozym 435**.**
(Tolba, et al., 2013) (Ceschel et al., 2002) (Celli et al., 2004) (Demestre et al., 2009)	**Poor solubility and bioavailability** Tolba et al., 2013 documented that CAPE is freely soluble in organic solvents ethanol, methanol, acetone, DMSO (10mg/ml) while **practically insoluble in water.** Collectively the literature available for pharmacokinetic properties of CAPE demonstrated its poor bioavailability. Ceschel et al., 2002 investigated diffusion and permeation of CAPE through porcine buccal mucosa using Franz diffusion cells. The results of study indicated high permeability coefficient for CAPE as compared that of other constituents of propolis. Celli et al., 2004 developed liquid chromatographic method for determination of CAPE in plasma and urine. The study reported **low but rapid absorption and excretion of CAPE after oral administration** in rats which confers its **short half-life.** Wang et al., 2009 studied pharmacokinetics of CAPE upon intravenous administration. The study disclosed that plasma concentration time curve for CAPE showed fast distribution phase with relatively slow elimination phase. Further the results demonstrated that area under curve (AUC) for CAPE increased at proportion greater than the rise in CAPE dose from 5 to 20 mg/kg. Demestreet al., 2009 reported that existing lacuna of clinical studies for CAPE was due to its **poor bioavailability / poor aqueous solubility**. Therefore, CAPE based propolis extract (Bio 30) was prepared using water miscible lipids to improve the solubility and bioavailability of CAPE. The extract successfully regulated growth of human neurofibromatosis (NF) tumor

	xenografts in mice.

Few of the patents or applications covering compositions and method of use of CAPE as listed in table 1.9.1 as follows:

Table 1.9.1. Patent literature for CAPE

Patents/ application No.	Description
US 6,689,811	Discloses method of potentiating radiation therapy by administering CAPE
US 6,696,484	Discloses pharmaceutical compositions containing CAPE for treatment of androgen related disorder
US 6,576,660	Discloses process of inhibiting 5α reductase activity using CAPE
US 5,981,583	Discloses method of inhibiting activation of nuclear transcription factor NF-kappa-β in cells using CAPE
EP1461025	Discloses use of CAPE for the treatment of parkinson's disease
EP2420289	Discloses composition comprising CAPE with other flavonoids like catechin, kaemferol and myricetin for treatment and/ or prevention.

1.4. NEED OF WORK:

The literature revealed necessity of work to be initiated for these apiproducts is addressed as follows:

For Bee Pollen:

- Previous scientific reports as discussed in section 1.1.3B reveal that several studies on chemical composition, palyonological investigations and benefits of bee pollen from different geographical regions like Australia Brazil, China, Chile, Portugal, South Africa, Sonoran Desert-USA have been reported. However, to our knowledge there is lacuna of studies examining the composition or benefits of bee pollen from Indian sources.
- The literature highlights another fact that despite of high nutritional content documented for bee pollen, ambiguities have been observed about digestibility of pollen by humans. It is documented that only a portion of pollen nutrients is assimilated by humans (Roulston and Cane, 2000, Campos et al., 2010). In general, the pollen structure is composed of nutrient rich cytoplasm enclosed within tough pollen wall or coat consisting of cellulosic inner layer 'intine' and external layer 'exine' made of sporopollenin. The ridged sporopollenin matrix composition of coat makes the pollen rigid thereby resists its decay and digestion, due to which most nutritional qualities of pollen pass through gut without being absorbed (Franchi et al., 1997, Roulston and Cane, 2000, Diego-Taboada et al., 2014). The resistant pollen wall structure hinders availability of engulfed nutrients for digestion and assimilation, thereby limits the usefulness of bee pollen for human consumption. This probed a hypothesis that cracking of pollen or pollen coat would improve availability of engulfed nutrients for digestion (Roulston and Cane, 2000, Campos et al., 2010). Literature shows that several chemical, mechanical and biological methods have been attempted for breaking the pollen wall (table 1.7, section B). The success of such methods is limited on account of associated drawbacks which include, degradation of nutrients by enzymatic or chemical treatments, need for sophisticated instrumentation, higher costs involved for mechanical techniques, etc.

Therefore there is need of an effective method for processing of bee pollen to obtain ruptured pollen with better availability of nutrients.

- Based on literature disclosing the rich polyphenols content and antioxidant effects, bee pollen are claimed to be a useful food or dietary supplement for sportspeople and athletes (Campos et al., 2010), however very few scientific evidences are available to support this claim (Maughan and Evans, 1982). In this context, there is need to ascertain the beneficial role of bee pollen in preclinical and clinical investigations.

For Propolis:

- The literature in sections1.1.3C and 1.3.2 reveal wide therapeutic potential of propolis in terms of array of pharmacological activities, most of which have been ascribed to the flavonoid and other polyphenolic constituents of propolis (Marcucci, 1995, Coneac et al., 2008, da Silva et al., 2013, Pellati et al., 2013).
- Despite of the wide therapeutic potential of propolis, low aqueous solubility, complex waxy, resinous nature and sticky consistency crafts a major difficulty in its extraction and processing. Large number of scientific reports state poor bioavailability of propolis constituents due to their low aqueous solubility (Metzner et al., 1978, Demestre et al., 2009, Tolba et al., 2013). This limits practical and commercial applications of propolis.
- Several techniques reported for extraction of active constituents from propolis includes conventional maceration, heat reflux extraction, ultrasonic extraction, super critical extraction, etc. The conventional methodologies suffer from drawbacks such as time consuming, require large amounts of organic solvents, cost on account of sophisticated instrumentation (eg: super critical extraction), etc.
- Water, the most “green” solvent, has been reported to dissolve only a small portion of propolis constituents, (about 10% of its weight) due to poor aqueous solubility of the flavonoids, phenolics and waxes (Margeretha et al., 2012). In order to achieve maximum extraction of biologically active constituents especially the flavonoids, propolis needs to be extracted using organic solvents like ethanol, methanol, acetone,

chloroform, etc (Cvek et al., 2007, Jang et al., 2009). However such solvents suffer from problem of volatility and flammability making the extraction process thermally unsafe. Moreover the extracts produced may have limited water solubility and are difficult to disperse in aqueous fluids in the body.

- Few researchers have used hydro-alcoholic solvents for extraction of active phenolics from raw propolis (Bankova et al., 2002a, Park and Ikegaki, 1998, Cunha et al., 2004), however extracts produced thereby may invite a concern about residual organic solvents leading to detrimental effects and toxicity upon ingestion.

In light of these facts, for potentiation of propolis there is need of green method which can improve extraction of bioactive flavonoids without use of toxic organic solvents.

For CAPE:

- The literature discussed in section 1.3.3 confirms wide array of pharmacological activities for CAPE mainly anticancer, antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, antiviral, neuro-protective, etc. Although numerous preclinical studies demonstrate effectiveness of CAPE, its therapeutic utility is limited on account of its poor bioavailability which is ascribed to its poor aqueous solubility (Demestre et al., 2009, Celli et al., 2004, Tolba et al., 2013).
- This has reflected in lacuna of clinical studies for CAPE in literature. Therefore there is need of efforts to improve the aqueous solubility of CAPE which in turn may modulate its bioavailability.
- Most of the documented scientific reports focus on chemical synthesis and biological evaluations of CAPE or its derivatives, however, a systematic analysis of its crystal structure landscape directed towards improving its physicochemical properties is not known in literature. There exists lacuna of reports on the crystal forms of CAPE either as single or multicomponent forms.

References:

Ajibola A, Chamunorwa JP and Erlwanger KH (2012). Nutraceutical values of natural honey and its contribution to human health and wealth. *Nutr Metab (Lond),* 9**:**61.

Al Somal N, Coley K, Molan P and Hancock B (1994). Susceptibility of Helicobacter pylori to the antibacterial activity of manuka honey. *Journal of the Royal Society of Medicine,* 87**:**9-12.

Ali M (2012). Studies on bee venom and its medical uses. *Int J Adv Res Technol,* 1**:**69-83.

Aliyazicioglu Y, Deger O, Ovali E, Barlak Y, Hosver I, Tekelioglu Y and Karahan SC (2005). Effects of Turkish pollen and propolis extracts on respiratory burst for K-562 cell lines. *International immunopharmacology,* 5**:**1652-57.

Almaraz AN, Campos MG, Avila RJA, Naranjo JN, Herrera CJ and Gonzalez VLS (2004). Variability of antioxidant activity among honeybee-collected pollen of different botanical origin. *Interciencia Caracas,* 29**:**574-78.

Amaya MM (2009). Floral constancy in bees: a revision of theories and a comparison with other pollinators. *Revista Colombiana de Entomología,* 35**:**206-16.

Anauate NC, Anido AA, Leegoy HR, Matsumoto R, Alonso RCB, Marcucci MC, Paulino N and Bretz WA (2014). Randomized, double-blind, placebo-controlled clinical trial on the effects of propolis and chlorhexidine mouthrinses on gingivitis. *Brazilian dental science,* 17**:**11.

Araujo MA, Liberio SA, Guerra RN, Ribeiro MNS and Nascimento FR (2012). Mechanisms of action underlying the anti-inflammatory and immunomodulatory effects of propolis: a brief review. *Revista Brasileira de Farmacognosia,* 22**:**208-19.

Bandyopadhyay S, Lee M, Sivaraman J and Chatterjee C (2013). Model membrane interaction and DNA-binding of antimicrobial peptide Lasioglossin II derived from bee venom. *Biochemical and biophysical research communications,* 430**:**1-6.

Bankova V (2005). Recent trends and important developments in propolis research. *Evidence-based complementary and alternative medicine,* 2**:**29-32.

Bankova V (2009). Chemical diversity of propolis makes it a valuable source of new biologically active compounds. *Journal of ApiProduct and ApiMedical Science,* 1**:**23-28.

Bankova V, Dyulgerov A, Popov S and Marekov N (1987). A GC/MS study of the propolis phenolic constituents. *Zeitschrift für Naturforschung C,* 42**:**147-51.

Bankova V, Galabov A, Antonova D, Vilhelmova N and Di Perri B (2014a). Chemical composition of Propolis Extract ACF® and activity against herpes simplex virus. *Phytomedicine,* 21**:**1432-38.

Bankova V, Popova M, Bogdanov S and Sabatini A-G (2002a). Chemical composition of European propolis: expected and unexpected results. *Zeitschrift fur Naturforschung C,* 57**:**530-33.

Bankova V, Popova M, Bogdanov S and Sabatini AG (2002b). Chemical composition of European propolis: expected and unexpected results. *Zeitschrift für Naturforschung C,* 57**:**530-33.

Bankova V, Popova M and Trusheva B (2014b). Propolis volatile compounds: chemical diversity and biological activity: a review. *Chemistry Central Journal,* 8**:**28.

Bankova VS, De Castro SL and Marcucci MC (2000). Propolis: recent advances in chemistry and plant origin. *Apidologie,* 31**:**3-16.

Banskota AH, Tezuka Y and Kadota S (2001). Recent progress in pharmacological research of propolis. *Phytotherapy Research,* 15**:**561-71.

Bharti AC and Aggarwal BB (2002). Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochemical pharmacology,* 64**:**883-88.

Biscaia D and Ferreira SR (2009). Propolis extracts obtained by low pressure methods and supercritical fluid extraction. *The Journal of Supercritical Fluids,* 51**:**17-23.

Bogdanov S (2009). Beeswax: production, properties, composition and control. *Beeswax book. Muehlethurnen, Switzerland: Bee Product Science Publishing. p***:**1-17.

Bogdanov S (2014). Pollen: Production, Nutrition and Health: A Review. *Bee Product Science*.

Bogdanov S, Jurendic T, Sieber R and Gallmann P (2008). Honey for nutrition and health: a review. *Journal of the American College of Nutrition,* 27**:**677-89.

Boisard S, Le Ray AM, Landreau A, Kempf M, Cassisa V, Flurin C and Richomme P (2015). Antifungal and antibacterial metabolites from a French poplar type propolis. *Evidence-Based Complementary and Alternative Medicine,* 2015**:**1-10.

Bonvehí JS and Gutierrez AL (2012). The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain). *World Journal of Microbiology and Biotechnology,* 28**:**1351-58.

Boyanova L, Derejian S, Koumanova R, Katsarov N, Gergova G, Mitov I, Nikolov R and Krastev Z (2003). Inhibition of Helicobacter pylori growth in vitro by Bulgarian propolis: preliminary report. *Journal of medical microbiology,* 52**:**417-19.

Boyanova L, Gergova G, Nikolov R, Derejian S, Lazarova E, Katsarov N, Mitov I and Krastev Z (2005). Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. *Journal of medical microbiology,* 54**:**481-83.

Bradbear N (2009). Bees and their role in forest livelihoods: a guide to the services provided by bees and the sustainable harvesting, processing and marketing of their products. *Non-wood Forest Products*.

Brady N, Molan P and Harfoot C (1996). The sensitivity of dermatophytes to the antimicrobial activity of manuka honey and other honey. *Pharmacy and Pharmacology Communications,* 2**:**471-73.

Brooks J and Shaw G (1978). Sporopollenin: a review of its chemistry, palaeochemistry and geochemistry. *Grana,* 17**:**91-97.

Burczyk J and Dworzanski J (1988). Comparison of sporopollenin-like algal resistant polymer from cell wall of Botryococcus, Scenedesmus and Lycopodium clavatum by GC-pyrolysis. *Phytochemistry,* 27**:**2151-53.

Burdock G (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology,* 36**:**347-63.

Burke TRJ, Fesen M, Mazumder A, Yung J, Wang J, Carothers AM, Grunberger D, Driscoll J, Pommier Y and Kohn K (1995). Hydroxylated aromatic inhibitors of HIV-1 integrase. *Journal of medicinal chemistry,* 38**:**4171-78.

Campos M, Frigerio C, Lopes J and Bogdanov S (2010). What is the future of Bee-Pollen. *J ApiProduct ApiMedical Sci,* 2**:**131-44.

Campos M, Markham K and Proença Da Cunha A (1997a). Quality assessment of bee-pollens using flavonoid/phenolic profiles. *Polyphenol Communications,* 96**:**53-54.

Campos M, Markham KR, Mitchell KA and Da Cunha AP (1997b). An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. *Phytochemical Analysis,* 8**:**181-85.

Campos MG, Bogdanov S, De Almeida-Muradian LB, Szczesna T, Mancebo Y, Frigerio C and Ferreira F (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research,* 47**:**154-61.

Campos MG, Webby RF, Markham KR, Mitchell KA and Da Cunha AP (2003). Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *Journal of agricultural and food chemistry,* 51**:**742-45.

Carlos A, David H and Carmen G (2011). Role of honey polyphenols in health. *Journal of ApiProduct and ApiMedical Science,* 3**:**141-59.

Carpes S, Mourao G, De Alencar S and Masson M (2009). Chemical composition and free radical scavenging activity of Apis mellifera bee pollen from Southern Brazil. *Brazilian Journal of Food Technology,* 12**:**220-29.

Carpes ST, Begnini R, Alencar SMD and Masson ML (2007). Study of preparations of bee pollen extracts, antioxidant and antibacterial activity. *Ciência e Agrotecnologia,* 31**:**1818-25.

Celli N, Mariani B, Dragani LK, Murzilli S, Rossi C and Rotilio D (2004). Development and validation of a liquid chromatographic–tandem mass spectrometric method for the determination of caffeic acid phenethyl ester in rat plasma and urine. *Journal of Chromatography B,* 810**:**129-36.

Ceschel G, Maffei P, Sforzini A, Borgia SL, Yasin A and Ronchi C (2002). In vitro permeation through porcine buccal mucosa of caffeic acid phenetyl ester (CAPE) from a topical mucoadhesive gel containing propolis. *Fitoterapia,* 73**:**S44-S52.

Chen MF, Wu CT, Chen YJ and Chen WC (2004). Cell killing and radiosensitization by caffeic acid phenethyl ester (CAPE) in lung cancer cells. *Journal of radiation research,* 45**:**253-60.

Chen MJ, Chang WH, Lin CC, Liu CY, Wang TE, Chu CH, Shih SC and Chen YJ (2008). Caffeic acid phenethyl ester induces apoptosis of human pancreatic cancer cells involving caspase and mitochondrial dysfunction. *Pancreatology,* 8**:**566-76.

Choi EM (2007). Antinociceptive and antiinflammatory activities of pine (Pinus densiflora) pollen extract. *Phytotherapy research,* 21**:**471-75.

Choudhari MK, Haghniaz R, Rajwade JM and Paknikar KM (2013). Anticancer activity of Indian stingless bee propolis: an in vitro study. *Evidence-Based Complementary and Alternative Medicine,* 2013**:**1-10.

Cihan YB, Ozturk A and Gokalp SS (2013). Protective Role of Royal Jelly Against Radiation-Induced Oxidative Stress in Rats. *International Journal of Hematology and Oncology,* 23**:**79-87.

Cikman O, Taysi S, Gulsen MT, Demir E, Akan M, Diril H, Kiraz HA, Karaayvaz M and Tarakcioglu M (2015). The Radio-protective effects of Caffeic Acid Phenethyl Ester and Thymoquinone in rats exposed to total head irradiation. *Wiener klinische Wochenschrift,* 127**:**103-08.

Cocan O, Marghitas L, Dezmirean D and Laslo L (2005). Composition and biological activities of bee pollen: review. *Bulletin of the University of Agricultural Science and Veterinary Medicine,* 61**:**221-26.

Coneac G, Gafiţanu E, Hadaruga D, Hadaruga N, Pinzaru I, Bandur G, Ursica L, Paunescu V and Gruia A (2008). Flavonoid contents of propolis from the west side of Romania and correlation with the antioxidant activity. *Chemical Bulletin "Politehnica" University Timisoara,* 53**:**56-60.

Cunha I, Sawaya AC, Caetano FM, Shimizu MT, Marcucci MC, Drezza FT, Povia GS and Carvalho PDO (2004). Factors that influence the yield and composition of Brazilian propolis extracts. *Journal of the Brazilian Chemical Society,* 15**:**964-70.

Cvek J, Medic-Saric M, Jasprica I, Zubcic S, Vitali D, Mornar A, Vedrina-Dragojevic I and Tomic S (2007). Optimisation of an extraction procedure and chemical characterisation of Croatian propolis tinctures. *Phytochemical Analysis,* 18**:**451-59.

Da Silva FC, Da Fonseca CR, De Alencar SM, Thomazini M, De Carvalho Balieiro JC, Pittia P and Favaro-Trindade CS (2013). Assessment of production efficiency, physicochemical properties and storage stability of spray-dried propolis, a natural food additive, using gum Arabic and OSA starch-based carrier systems. *Food and Bioproducts Processing,* 91**:**28-36.

Daleprane JB and Abdalla DS (2013). Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. *Evidence-based complementary and alternative medicine,* 2013.

Dalio JS (2012). Cannabis sativa-An Important Subsistence Pollen Source for Apis mellifera. *J Phar Biol Sci,* 1**:**1-3.

Demestre M, Messerli S, Celli N, Shahhossini M, Kluwe L, Mautner V and Maruta H (2009). CAPE (caffeic acid phenethyl ester)-based propolis extract (Bio 30)

suppresses the growth of human neurofibromatosis (NF) tumor xenografts in mice. *Phytotherapy Research,* 23**:**226-30.

Diego-Taboada A, Beckett ST, Atkin SL and Mackenzie G (2014). Hollow pollen shells to enhance drug delivery. *Pharmaceutics,* 6**:**80-96.

Doo AR, Kim ST, Kim SN, Moon W, Yin CS, Chae Y, Park HK, Lee H and Park HJ (2010). Neuroprotective effects of bee venom pharmaceutical acupuncture in acute 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced mouse model of Parkinson's disease. *Neurological research,* 32**:**88-91.

Du Lianxiang QWQL (2004). Study on Solid-state Fermentation Conditions of the Bee Pollen. *Food and Fermentation Industries,* 12**:**015.

Ediriweera E and Premarathna N (2012). Medicinal and cosmetic uses of Bee's Honey–A review. *Ayu,* 33**:**178.

Efem S (1988). Clinical observations on the wound healing properties of honey. *British journal of Surgery,* 75**:**679-81.

El Asely AM, Abbass AA and Austin B (2014). Honey bee pollen improves growth, immunity and protection of Nile tilapia (Oreochromis niloticus) against infection with Aeromonas hydrophila. *Fish and shellfish immunology,* 40**:**500-06.

El Nekeety AA, El-Kholy W, Abbas NF, Ebaid A, Amra HA and Abdel-Wahhab MA (2007). Efficacy of royal jelly against the oxidative stress of fumonisin in rats. *Toxicon,* 50**:**256-69.

Erdogan S, Ates B, Durmaz G, Yilmaz I and Seckin T (2011). Pressurized liquid extraction of phenolic compounds from Anatolia propolis and their radical scavenging capacities. *Food and Chemical Toxicology,* 49**:**1592-97.

Escuredo O, Míguez M, Fernández-González M and Seijo MC (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food chemistry,* 138**:**851-56.

Espelie KE, Loewus FA, Pugmire RJ, Woolfenden WR, Baldi BG and Given PH (1989). Structural analysis of Lilium longiflorum sporopollenin by 13 C NMR spectroscopy. *Phytochemistry,* 28**:**751-53.

Eterf Oskouei T and Najafi M (2013). Traditional and modern uses of natural honey in human diseases: a review. *Iranian journal of basic medical sciences,* 16**:**731.

Fadillioglu E, Oztas E, Erdogan H, Yagmurca M, Sogut S, Ucar M and Irmak MK (2004). Protective effects of caffeic acid phenethyl ester on doxorubicin-induced cardiotoxicity in rats. *Journal of Applied Toxicology,* 24**:**47-52.

Fang K, Wang Y, Yu T, Zhang L, Baluška F, Šamaj J and Lin J (2008). Isolation of de-exined pollen and cytological studies of the pollen intines of Pinus bungeana Zucc. Ex Endl. and Picea wilsonii Mast. *Flora-Morphology, Distribution, Functional Ecology of Plants,* 203**:**332-40.

Farooqui AA (2012). Beneficial effects of propolis on neurological disorders. Phytochemicals, Signal Transduction, and Neurological Disorders. Springer, 301-22.

Feas X, Vazquez-Tato MP, Estevinho L, Seijas JA and Iglesias A (2012). Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules,* 17**:**8359-77.

Fíla J, Capkova V, Fecikova J and Honys D (2011). Impact of homogenization and protein extraction conditions on the obtained tobacco pollen proteomic patterns. *Biologia Plantarum,* 55**:**499-506.

Fokt H, Pereira A, Ferreira A, Cunha A and Aguiar C (2010). How do bees prevent hive infections? The antimicrobial properties of propolis. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology,* 1**:**481-4493.

Franchi G, Franchi G and Pompella A (1997). Microspectroscopic evaluation of digestibilty of pollen grains. *Plant Foods for Human Nutrition,* 50**:**115-26.

Frankel S, Robinson G and Berenbaum M (1998). Antioxidant capacity and correlated characteristics of 14 unifloral honeys. *Journal of Apicultural Research,* 37**:**27-31.

Freire KR, Lins A, Dórea MC, Santos FA, Camara CA and Silva T (2012). Palynological origin, phenolic content, and antioxidant properties of honeybee-collected pollen from Bahia, Brazil. *Molecules,* 17**:**1652-64.

Fujiwara S, Imai J, Fujiwara M, Yaeshima T, Kawashima T and Kobayashi K (1990). A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. *Journal of biological chemistry,* 265**:**11333-37.

Gandhi GP, "Honey a potential item for export". Market Survey. 2006.

Gasic S, Vucevic D, Vasilijic S, Antunovic M, Chinou I and Colic M (2007). Evaluation of the immunomodulatory activities of royal jelly components in vitro. *Immunopharmacology and immunotoxicology,* 29**:**521-36.

Geyman JP (1993). Anaphylactic reaction after ingestion of bee pollen. *The Journal of the American Board of Family Practice/American Board of Family Practice,* 7**:**250-52.

Gharzouli K, Amira S, Gharzouli A and Khennouf S (2002). Gastroprotective effects of honey and glucose-fructose-sucrose-maltose mixture against ethanol-, indomethacin-, and acidified aspirin-induced lesions in the rat. *Experimental and toxicologic pathology,* 54**:**217-21.

Gheldof N and Engeseth NJ (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *Journal of agricultural and food chemistry,* 50**:**3050-55.

Gheldof N, Wang X-H and Engeseth NJ (2002). Identification and quantification of antioxidant components of honeys from various floral sources. *Journal of agricultural and food chemistry,* 50**:**5870-77.

Ghisalberti E (1979). Propolis: a review (honey-bees). *Bee world,* 60**:**59-84.

Gocer H and Gulin İ (2011). Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties. *International journal of food sciences and nutrition,* 62**:**821-25.

Greenberger PA and Flais MJ (2001). Bee pollen-induced anaphylactic reaction in an unknowingly sensitized subject. *Annals of allergy, asthma and immunology,* 86**:**239-42.

Grunberger D, Banerjee R, Eisinger K, Oltz E, Efros L, Caldwell M, Estevez V and Nakanishi K (1988). Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. *Experientia,* 44**:**230-32.

Gupta RK and Stangaciu S (2014). Apitherapy: holistic healing through the honeybee and bee products in countries with poor healthcare system. Beekeeping for poverty alleviation and livelihood security. Springer, 413-46.

Haffejee I and Moosa A (1985). Honey in the treatment of infantile gastroenteritis. *BMJ,* 290**:**1866-67.

He YT, Wang T, Yu Y and Wang YP (2006). Studies on Wall-breaking Methods of Corn Pollen. *Food Science,* 10**:**067.

Inoue SI, Koya MS, Ushio S, Iwaki K, Ikeda M and Kurimoto M (2003). Royal Jelly prolongs the life span of C3H/HeJ mice: correlation with reduced DNA damage. *Experimental gerontology,* 38**:**965-69.

Izuta H, Shimazawa M, Tsuruma K, Araki Y, Mishima S and Hara H (2009). Bee products prevent VEGF-induced angiogenesis in human umbilical vein endothelial cells. *BMC complementary and alternative medicine,* 9**:**45.

Jang MJ, Sheu SR, Wang CC, Yeh YL and Sung KH 2009. Optimization analysis of the experimental parameters on the extraction process of propolis. *Proceedings of the International MultiConference of Engineers and Computer Scientists.* Citeseer.

Jeffrey AE and Echazarreta CM (1996). Medical uses of honey. *Rev Biomed,* 7**:**43-49.

Kai H, Obuchi M, Yoshida H, Watanabe W, Tsutsumi S, Park YK, Matsuno K, Yasukawa K and Kurokawa M (2014). In vitro and in vivo anti-influenza virus activities of flavonoids and related compounds as components of Brazilian propolis (AF-08). *Journal of Functional Foods,* 8**:**214-23.

Kamakura M (2011). Royalactin induces queen differentiation in honeybees. *Nature,* 473**:**478-83.

Karan B, Yashwant S and Parampal S (2013). Constraints of apiculture in India. *Inter. J. Life Sci. Res,* 1**:**1-4.

Kerman M, Kanter M, Coşkun KK, Erboga M and Gurel A (2012). Neuroprotective effects of Caffeic acid phenethyl ester on experimental traumatic brain injury in rats. *Journal of molecular histology,* 43**:**49-57.

Ketkar SS, Rathore AS, Lohidasan S, Rao L, Paradkar AR and Mahadik KR (2014). Investigation of the nutraceutical potential of monofloral Indian mustard bee pollen. *Journal of integrative medicine,* 12**:**379-89.

Khacha AS, Tragoolpua K, Chantawannakul P and Tragoolpua Y (2013). Antioxidant and anti-cancer cell proliferation activity of propolis extracts from two extraction methods. *Asian Pacific journal of cancer prevention,* 14**:**6991-95.

Kishimoto N, Kakino Y, Iwai K and Fujita T (2005a). Chlorogenate hydrolase-catalyzed synthesis of hydroxycinnamic acid ester derivatives by

transesterification, substitution of bromine, and condensation reactions. *Applied microbiology and biotechnology,* 68**:**198-202.

Kishimoto N, Kakino Y, Iwai K, Mochida K and Fujita T (2005b). In vitro antibacterial, antimutagenic and anti-influenza virus activity of caffeic acid phenethyl esters. *Biocontrol Science,* 10**:**155-61.

Koh PS, Seo BK, Cho NS, Park HS, Park DS and Baek YH (2013). Clinical effectiveness of bee venom acupuncture and physiotherapy in the treatment of adhesive capsulitis: a randomized controlled trial. *Journal of Shoulder and Elbow Surgery,* 22**:**1053-62.

Komosinska VK, Olczyk P, Kaźmierczak J, Mencner L and Olczyk K (2015). Bee Pollen: Chemical Composition and Therapeutic Application. *Evidence-Based Complementary and Alternative Medicine,* 2015.

Kovacik L, Plitzko JM, Grote M and Reichelt R (2009). Electron tomography of structures in the wall of hazel pollen grains. *Journal of structural biology,* 166**:**263-71.

Koya MS, Okamoto I, Ushio S, Iwaki K, Ikeda M and Kurimoto M (2004). Identification of a collagen production-promoting factor from an extract of royal jelly and its possible mechanism. *Bioscience, biotechnology, and biochemistry,* 68**:**767-73.

Krell R (1996). Value-added products from beekeeping. Food and Agriculture Org. UN. Rome.

Kubina RW, Kabała-Dzik A, Dziedzic A, Bielec B, Wojtyczka RD, Bułdak RJ, Wyszyńska M, Stawiarska-Pieta B and Szaflarska-Stojko E (2015). The Ethanol Extract of Polish Propolis Exhibits Anti-Proliferative and/or Pro-Apoptotic Effect on HCT 116 Colon Cancer and Me45 Malignant Melanoma Cells In Vitro Conditions. *Advances in Clinical and Experimental Medicine,* 24**:**203-12.

Kujumgiev A, Bankova V, Igantova A and Popov S (1993). Antibacterial activity of propolis, some of its components and their analogs. *Pharmazie,* 48**:**785-86.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R and Popov S (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *Journal of ethnopharmacology,* 64**:**235-40.

Kumazawa S, Hamasaka T and Nakayama T (2004). Antioxidant activity of propolis of various geographic origins. *Food Chemistry,* 84**:**329-39.

Kuo HC, Kuo WH, Lee YJ, Lin WL, Chou FP and Tseng TH (2006). Inhibitory effect of caffeic acid phenethyl ester on the growth of C6 glioma cells in vitro and in vivo. *Cancer letters,* 234**:**199-208.

Kuo YY, Lin HP, Huo C, Su LC, Yang J, Hsiao PH, Chiang HC, Chung CJ, Wang HD and Chang JY (2013). Caffeic acid phenethyl ester suppresses proliferation and survival of TW2. 6 human oral cancer cells via inhibition of Akt signaling. *International journal of molecular sciences,* 14**:**8801-17.

Lagouri V, Prasianaki D and Krysta F (2014). Antioxidant properties and phenolic composition of Greek propolis extracts. *International Journal of Food Properties,* 17**:**511-22.

Lahouel M, Boutabet K, Kebsa W and Alyane M (2010). Polyphenolic fractions of Algerian propolis reverses doxorubicin induced acute renal oxidative stress. *Afr J Pharm Pharmacol,* 4**:**712-20.

Leblanc BW, Davis OK, Boue S, Delucca A and Deeby T (2009). Antioxidant activity of Sonoran Desert bee pollen. *Food Chemistry,* 115**:**1299-305.

Lee JA, Son MJ, Choi J, Yun K-J, Jun JH and Lee MS (2014). Bee venom acupuncture for rheumatoid arthritis: a systematic review protocol. *BMJ open,* 4**:**e004602.

Leja M, Mareczek A, Wyżgolik G, Klepacz-Baniak J and Czekońska K (2007). Antioxidative properties of bee pollen in selected plant species. *Food Chemistry,* 100**:**237-40.

Li X, Huang C and Xue Y (2013). Contribution of lipids in honeybee (Apis mellifera) royal jelly to health. *Journal of medicinal food,* 16**:**96-102.

Liao HF, Chen YY, Liu JJ, Hsu ML, Shieh HJ, Liao HJ, Shieh CJ, Shiao MS and Chen YJ (2003). Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis. *Journal of agricultural and food chemistry,* 51**:**7907-12.

Lin HP, Lin CY, Huo C, Hsiao PH, Su LC, Jiang SS, Chan TM, Chang CH, Chen LT and Kung HJ (2015). Caffeic acid phenethyl ester induced cell cycle arrest and growth inhibition in androgen-independent prostate cancer cells via regulation of Skp2, p53, p21Cip1 and p27Kip1. *Oncotarget,* 6**:**6684-707.

Liu W and Wang X (2004). Extraction of flavone analogues from propolis with ultrasound. *Food Sci (China),* 25**:**35-39.

Loewus FA, Baldi BG, Franceschi VR, Meinert LD and Mccollum JJ (1985). Pollen sporoplasts: dissolution of pollen walls. *Plant physiology,* 78**:**652-54.

Lombardo AM, Garcia CAM, Gamiz GL and Cruces BC (2012). Determination of quinolones of veterinary use in bee products by ultra-high performance liquid chromatography–tandem mass spectrometry using a QuEChERS extraction procedure. *Talanta,* 93**:**193-99.

Ls VK (2014). Propolis in dentistry and oral cancer management. *North American journal of medical sciences,* 6**:**250.

Lv H, Wang X, He Y, Wang H and Suo Y (2015). Identification and quantification of flavonoid aglycones in rape bee pollen from Qinghai-Tibetan Plateau by HPLC-DAD-APCI/MS. *Journal of Food Composition and Analysis,* 38**:**49-54.

M De Figueiredo S, A Nogueira-Machado J, De M Almeida B, Rl Abreu S, As De Abreu J, Av Filho S, S Binda N and B Caligiorne R (2014). Immunomodulatory properties of green propolis. *Recent patents on endocrine, metabolic and immune drug discovery,* 8**:**85-94.

Maeda Y, Loughrey A, Earle JP, Millar BC, Rao JR, Kearns A, Mcconville O, Goldsmith CE, Rooney PJ and Dooley JS (2008). Antibacterial activity of honey against community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA). *Complementary Therapies in Clinical Practice,* 14**:**77-82.

Marcucci MC (1995). Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie,* 26**:**83-99.

Margaoan R, MăRghitaş L, Dezmirean D, Mihai C and Bobis O (2010). Bee collected pollen-general aspects and chemical composition. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies,* 67**:**254-59.

Margeretha I, Suniarti DF, Herda E and Mas'ud ZA (2012). Optimization and comparative study of different extraction methods of biologically active components of Indonesian propolis Trigona spp. *J Nat Prod,* 5**:**233-42.

Marghitas LA, Dezmirean DS and Bobis O (2013). Important developments in Romanian propolis research. *Evidence-Based Complementary and Alternative Medicine,* 2013.

Marquele FD, Di Mambro VM, Georgetti SR, Casagrande R, Valim YM and Fonseca MJV (2005). Assessment of the antioxidant activities of Brazilian extracts of propolis alone and in topical pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis,* 39**:**455-62.

Maruyama H, Sakamoto T, Araki Y and Hara H (2010). Anti-inflammatory effect of bee pollen ethanol extract from Cistus sp. of Spanish on carrageenan-induced rat hind paw edema. *BMC complementary and alternative medicine,* 10**:**30.

Maughan R and Evans S (1982). Effects of pollen extract upon adolescent swimmers. *British journal of sports medicine,* 16**:**142-45.

Meda A, Lamien CE, Millogo J, Romito M and Nacoulma OG (2004). Therapeutic uses of honey and honeybee larvae in central Burkina Faso. *Journal of ethnopharmacology,* 95**:**103-07.

Medeiros K, Figueiredo C, Figueredo T, Freire K, Santos F, Alcantara-Neves N, Silva T and Piuvezam M (2008). Anti-allergic effect of bee pollen phenolic extract and myricetin in ovalbumin-sensitized mice. *Journal of ethnopharmacology,* 119**:**41-46.

Medhi B, Puri A, Upadhyay S and Kaman L (2008). Topical application of honey in the treatment of wound healing: a metaanalysis. *JK Sci,* 10**:**166-69.

Mejías E and Montenegro G (2012). The antioxidant activity of Chilean honey and bee pollen produced in the Llaima Volcano's Zones. *Journal of Food Quality,* 35**:**315-22.

Metzner J, Bekemeier H, Schneidewind E and Wenzel U (1978). Pharmacokinetic studies of the propolis constituent pinocembrin in the rats. *Die Pharmazie,* 34**:**185-87.

Michaluart P, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, Mestre JR, Grunberger D, Sacks PG and Tanabe T (1999). Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. *Cancer research,* 59**:**2347-52.

Moita E, Sousa C, Andrade PB, Fernandes F, Pinho BR, Silva LR and Valentão P (2014). Effects of Echium plantagineum L. bee pollen on basophil degranulation: Relationship with metabolic profile. *Molecules,* 19**:**10635-49.

Morais M, Moreira L, Feás X and Estevinho LM (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology,* 49**:**1096-101.

More S, Ghorai N and Bera S 2010. Study on the selection of some local pollen plants in West Bengal, India as pollen brood feed in Apis cerana Fabr. Larvae by the worker bees, through meconia analysis and community ordination method of recovered pollen taxa. *Proceedings of the Zoological Society.* Springer.

Moret S, Purcaro G and Conte LS (2010). Polycyclic aromatic hydrocarbons (PAHs) levels in propolis and propolis-based dietary supplements from the Italian market. *Food chemistry,* 122**:**333-38.

Munstedt K, Hackethal A and Schmidt K (2005). Bee venom therapy, bee venom acupuncture or apipuncture--what is the evidence behind the various health claims? *American bee journal*.

Münstedt K, Wrobel D and Kalder M (2010). Efficacy of venom immunotherapy in beekeepers. *J Investig Allergol Clin Immunol,* 20**:**58-62.

Murtaza G, Karim S, Akram MR, Khan SA, Azhar S, Mumtaz A and Bin Asad MHH (2014a). Caffeic acid phenethyl ester and therapeutic potentials. *BioMed research international,* 2014.

Murtaza G, Karim S, Akram MR, Khan SA, Azhar S, Mumtaz A and Bin Asad MHH (2014b). Caffeic acid phenethyl ester and therapeutic potentials. *BioMed research international,* 2014**:**1-9.

Mutsaers M, Blitterswijk HV, Leven LVT, Kerkvliet J and Jan Van DW (2005). Bee products: properties, processing and marketing. Agromisa Foundation, Wageningen.

Naik DG, Mujumdar AM and Vaidya HS (2013a). Anti-inflammatory activity of propolis from Maharashtra, India. *Journal of Apicultural Research,* 52**:**35-43.

Naik DG, Vaidya HS and Namjoshi TP (2013b). Essential oil of Indian propolis: chemical composition and repellency against the honeybee apis florea. *Chemistry and biodiversity,* 10**:**649-57.

Nasuti C, Gabbianelli R, Falcioni G and Cantalamessa F (2006). Antioxidative and gastroprotective activities of anti-inflammatory formulations derived from chestnut honey in rats. *Nutrition Research,* 26**:**130-37.

Natarajan K, Singh S, Burke TR, Grunberger D and Aggarwal BB (1996). Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proceedings of the National Academy of Sciences,* 93**:**9090-95.

Ojeda CAJ, Hernandez MJ, Dominguez Z, Mercado RJN, Troncoso RR, Sanchez EA and Tiznado HME (2008). Utilization of caffeic acid phenethyl ester to control Alternaria alternata rot in tomato (*Lycopersicon esculentum* Mill.) fruit. *Journal of phytopathology,* 156**:**164-73.

Oktem F, Yilmaz HR, Ozguner F, Olgar S, Ayata A, Uzar E and Uz E (2006). Methotrexate-induced renal oxidative stress in rats: the role of a novel

antioxidant caffeic acid phenethyl ester. *Toxicology and industrial health,* 22**:**241-47.

Orsolic N (2012). Bee venom in cancer therapy. *Cancer and metastasis reviews,* 31**:**173-94.

Ozen S, Akyol O, Iraz M, Sogut S, Ozugurlu F, Ozyurt H, Odacı E and Yıldırım Z (2004). Role of caffeic acid phenethyl ester, an active component of propolis, against cisplatin-induced nephrotoxicity in rats. *Journal of Applied Toxicology,* 24**:**27-35.

Ozturk G, Ginis Z, Akyol S, Erden G, Gurel A and Akyol O (2012). The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers. *Eur Rev Med Pharmacol Sci,* 16**:**2064-68.

Pacini E and Hesse M (2005). Pollenkitt–its composition, forms and functions. *Flora-Morphology, Distribution, Functional Ecology of Plants,* 200**:**399-415.

Papotti G, Bertelli D, Bortolotti L and Plessi M (2012). Chemical and functional characterization of Italian propolis obtained by different harvesting methods. *Journal of agricultural and food chemistry,* 60**:**2852-62.

Park SG, Lee DY, Seo SK, Lee SW, Kim SK, Jung WK, Kang MS, Choi YH, Yea SS and Choi I (2008). Evaluation of anti-allergic properties of caffeic acid phenethyl ester in a murine model of systemic anaphylaxis. *Toxicology and applied pharmacology,* 226**:**22-29.

Park YK, Alencar SM and Aguiar CL (2002). Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry,* 50**:**2502-06.

Park YK and Ikegaki M (1998). Preparation of water and ethanolic extracts of propolis and evaluation of the preparations. *Bioscience, biotechnology, and biochemistry,* 62**:**2230-32.

Pascoal A, Rodrigues S, Teixeira A, Feás X and Estevinho LM (2014). Biological activities of commercial bee pollens: Antimicrobial, antimutagenic, antioxidant and anti-inflammatory. *Food and Chemical Toxicology,* 63**:**233-39.

Pavel CI, Marghitas LA, Bobis O, Dezmirean DS, Sapcaliu A, Radoi I and Madas MN (2011). Biological Activities of Royal Jelly-Review. *Scientific Papers Animal Science and Biotechnologies,* 44**:**108-18.

Pellati F, Prencipe FP, Bertelli D and Benvenuti S (2013). An efficient chemical analysis of phenolic acids and flavonoids in raw propolis by microwave-assisted extraction combined with high-performance liquid chromatography using the fused-core technology. *Journal of pharmaceutical and biomedical analysis,* 81**:**126-32.

Petelinc T, Polak T, Demšar L and Jamnik P (2013). Fractionation of phenolic compounds extracted from propolis and their activity in the yeast saccharomyces cerevisiae. *PloS one,* 8.

Polanski M, Okon K, Przybyło R and Frasik W (1997). Cardioprotective properties of hydrophilic pollen extract (HPE). *Polish journal of pathology: official journal of the Polish Society of Pathologists,* 49**:**109-12.

Ramadan M and Al-Ghamdi A (2012). Bioactive compounds and health-promoting properties of royal jelly: A review. *Journal of functional foods,* 4**:**39-52.

Roulston T and Cane JH (2000). Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution,* 222**:**187-209.

Ruan Z, Deng ZY, Wu LY, Xie MY and Wu MC (2008). Determination of Flavonoids Content in Rape Bee Pollen by HPLC and Effects of Six Cell Wall-breaking Techniques on Flavonoids Release from Rape Bee Pollen Cell. *Food Science,* 10**:**107.

Russo A, Cardile V, Sanchez F, Troncoso N, Vanella A and Garbarino J (2004). Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines. *Life sciences,* 76**:**545-58.

Rzepecka S A, Pilawa B, Ramos P and Stojko J (2012). Antioxidative properties of bee pollen extracts examined by EPR spectroscopy. *Journal of Apicultural Science,* 56**:**23-31.

Salles J, Cardinault N, Patrac V, Berry A, Giraudet C, Collin M-L, Chanet A, Tagliaferri C, Denis P and Pouyet C (2014). Bee Pollen Improves Muscle Protein and Energy Metabolism in Malnourished Old Rats through Interfering with the Mtor Signaling Pathway and Mitochondrial Activity. *Nutrients,* 6**:**5500-16.

Salomao K, Dantas A, Borba C, Campos L, Machado D, Aquino Neto F and Castro S (2004). Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis. *Letters in applied microbiology,* 38**:**87-92.

Salomao K, De Souza EM, Henriques-Pons A, Barbosa HS and De Castro SL (2011). Brazilian green propolis: effects in vitro and in vivo on Trypanosoma cruzi. *Evidence-Based Complementary and Alternative Medicine,* 2011.

Sampath Kumar K, Bhowmik D, Chiranjib B and Chandira M (2010). Medicinal uses and health benefits of Honey: An overview. *J Chem Pharm Res,* 2**:**385-95.

Schmidt J and Buchmann S (1992). Other products of the hive. *The Hive and the Honey Bee, Hamilton, Illinois***:**928-77.

Selamoglu ZS, Ozdemir I, Ciftci O, Gulhan MF and Savci A (2015). Antioxidant effect of ethanolic extract of propolis in liver of L-name treated rats. *Advances in clinical and experimental medicine,* 24**:**227-32.

Sforcin J (2007). Propolis and the immune system: a review. *Journal of ethnopharmacology,* 113**:**1-14.

Shen H, Yamashita A, Nakakoshi M, Yokoe H, Sudo M, Kasai H, Tanaka T, Fujimoto Y, Ikeda M and Kato N (2013). Inhibitory effects of caffeic acid phenethyl ester derivatives on replication of hepatitis C virus. *PloS one,* 8**:**e82299.

Shin BC, Kong JC, Park TY, Yang CY, Kang KW and Choi SM (2012). Bee venom acupuncture for chronic low back pain: A randomised, sham-controlled, triple-blind clinical trial. *European Journal of Integrative Medicine,* 4**:**e271-e80.

Silici S, Ekmekcioglu O, Eraslan G and Demirtas A (2009). Antioxidative effect of royal jelly in cisplatin-induced testes damage. *Urology,* 74**:**545-51.

Slijepcevic M, Nadzija M, Blazi PM and Boranic M 1979. Impact of the pollen contained in food on the reproduction of mice. *Proceedings. International Symposium on Apitherapy.*

Son S, Lobkowsky EB and Lewis BA (2001). Caffeic Acid Phenethyl Ester (CAPE): Synthesis and X-Ray Crstallographic Analysis. *Chemical and pharmaceutical bulletin,* 49**:**236-38.

Sousa C, Moita E, Valentão P, Fernandes F, Monteiro P and Andrade PB (2015). Effects of Colored and Noncolored Phenolics of Echium plantagineum L. Bee Pollen in Caco-2 Cells under Oxidative Stress Induced by tert-Butyl Hydroperoxide. *Journal of agricultural and food chemistry,* 63**:**2083-91.

Sreedevi P, Pillai G and Namboodiri A 1990. Solubility differences in sporopollenin of pollen and pollinial walls. Current Science ASSN.

Stevenson DE, Wibisono R, Jensen DJ, Stanley RA and Cooney JM (2006). Direct acylation of flavonoid glycosides with phenolic acids catalysed by Candida antarctica lipase B (Novozym 435®). *Enzyme and Microbial Technology,* 39**:**1236-41.

Suemaru K, Cui R, Li B, Watanabe S, Okihara K, Hashimoto K, Yamada H and Araki H (2008). Topical application of royal jelly has a healing effect for 5-fluorouracil-induced experimental oral mucositis in hamsters. *Methods and findings in experimental and clinical pharmacology,* 30**:**103-06.

Sugiyama T, Takahashi K and Mori H (2012). Royal jelly acid, 10-hydroxy-trans-2-decenoic acid, as a modulator of the innate immune responses. *Endocrine, Metabolic and Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine and Metabolic Disorders),* 12**:**368-76.

Swellam T, Miyanaga N, Onozawa M, Hattori K, Kawai K, Shimazui T and Akaza H (2003). Antineoplastic activity of honey in an experimental bladder cancer implantation model: in vivo and in vitro studies. *International journal of urology,* 10**:**213-19.

Tabuchi A, Li L and Cosgrove D (2011). Matrix solubilization and cell wall weakening by β-expansin (group-1 allergen) from maize pollen. *The Plant Journal,* 68**:**546-59.

Tang HW, Chan CH, Lin YT and Huang HY (2015). Anti-diabetic Effects of Propolis in High Fat Diet Fed-Streptozotocin-induced Type 2 Diabetic Rat. *The FASEB Journal,* 29**:**756.7.

Thomas D, Pal N and Rao KS (2002). Bee management and productivity of Indian honeybees. *Apiacta,* 3**:**1-15.

Tichy J and Novak J (2000). Detection of antimicrobials in bee products with activity against viridans streptococci. *The Journal of Alternative and Complementary Medicine,* 6**:**383-89.

Tokunaga KH, Yoshida C, Suzuki KM, Maruyama H, Futamura Y, Araki Y and Mishima S (2004). Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. *Biological and Pharmaceutical Bulletin,* 27**:**189-92.

Tolba MF, Azab SS, Khalifa AE, Abdel-Rahman SZ and Abdel-Naim AB (2013). Caffeic acid phenethyl ester, a promising component of propolis with a plethora of biological activities: A review on its anti-inflammatory, neuroprotective, hepatoprotective, and cardioprotective effects. *IUBMB life,* 65**:**699-709.

Torres D, Hollands I and Palacios E (1990). Effect of an alcoholic extract of propolis on the in vitro growth of Giardia lamblia. *Revista Cubana de Ciencias Veterinarias,* 21**:**15-19.

Townsend GF, Morgan JF, Tolnai S, Hazlett B, Morton HJ and Shuel R (1960). Studies on the in vitro antitumor activity of fatty acids I. 10-hydroxy-2-decenoic acid from royal jelly. *Cancer research,* 20**:**503-10.

Trusheva B, Trunkova D and Bankova V (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry central journal,* 1**:**13.

Tulloch AP (1980). Beeswax-composition and analysis. *Bee World,* 61**:**47-62.

Turner K, Nielsen B, O'connor C and Burton J (2006). Bee pollen product supplementation to horses in training seems to improve feed intake: a pilot study. *Journal of animal physiology and animal nutrition,* 90**:**414-20.

Velazquez C, Navarro M, Acosta A, Angulo A, Dominguez Z, Robles R, Robles-Zepeda R, Lugo E, Goycoolea F and Velazquez E (2007). Antibacterial and free-radical scavenging activities of Sonoran propolis. *Journal of applied microbiology,* 103**:**1747-56.

Vit P, Huq F, Barth O, Campo M, Perez PE, Tomas BF and Santos E (2015). Use of propolis in cancer research. *British Journal of Medicine and Medical Research,* 8**:**88-109.

Viuda MM, Ruiz NY, Fernandez LJ and Perez AJ (2008). Functional properties of honey, propolis, and royal jelly. *Journal of food science,* 73**:**R117-R24.

Wagh VD (2013). Propolis: a wonder bees product and its pharmacological potentials. *Advances in pharmacological sciences,* 2013.

Wang BJ, Lien YH and Yu ZR (2004). Supercritical fluid extractive fractionation–study of the antioxidant activities of propolis. *Food Chemistry,* 86**:**237-43.

Wang X, Wang H, Liu Y, You J and Suo Y (2009). Extraction of pollen lipids by SFE-CO2 and determination of free fatty acids by HPLC. *European journal of lipid science and technology,* 111**:**155-63.

Wesselius T, Heersema D, Mostert J, Heerings M, Admiraal-Behloul F, Talebian A, Van Buchem M and De Keyser J (2005). A randomized crossover study of bee sting therapy for multiple sclerosis. *Neurology,* 65**:**1764-68.

White J and Doner LW (1980). Honey composition and properties. *Beekeeping in the United States Agriculture Handbook,* 335**:**82-91.

Widjaja A, Yeh TH and Ju YH (2008). Enzymatic synthesis of caffeic acid phenethyl ester. *Journal of the Chinese Institute of Chemical Engineers,* 39**:**413-18.

Wiermann R and Gubatz S (1992). Pollen wall and sporopollenin. *International review of cytology***:**35-35.

Wittborn J, Rao K, El Ghazaly G and Rowley JR (1996). Substructure of spore and pollen grain exines in Lycopodium, Alnus, Betula, Fagus and Rhododendron: Investigation with Atomic Force and Scanning Tunnelling Microscopy. *Grana,* 35**:**185-98.

Wojcicki J, Samochowiec L, Dutkiewicz T and Tustanowski S (1991). The effect of pollen extracts on the endocrine function in rabbits. *Herba Polonica,* 3.

Wojcicki J, Samochowiec L and Kadhibowska D (1989). The influence of pollen extracts on biochemical disturbances in rats exposed to prolonged ethyl alcohol intake. *Herba Polonica,* 35**:**201.

Wu YD and Lou YJ (2007). A steroid fraction of chloroform extract from bee pollen of Brassica campestris induces apoptosis in human prostate cancer PC-3 cells. *Phytotherapy Research,* 21**:**1087-91.

Xiang D, Wang D, He Y, Xie J, Zhong Z, Li Z and Xie J (2006). Caffeic acid phenethyl ester induces growth arrest and apoptosis of colon cancer cells via the β-catenin/T-cell factor signaling. *Anti-cancer drugs,* 17**:**753-62.

Xu X, Sun L, Dong J and Zhang H (2009a). Breaking the cells of rape bee pollen and consecutive extraction of functional oil with supercritical carbon dioxide. *Innovative food science and emerging technologies,* 10**:**42-46.

Xu Y, Luo L, Chen B and Fu Y (2009b). Recent development of chemical components in propolis. *Frontiers of Biology in China,* 4**:**385-91.

Xuan H, Li Z, Yan H, Sang Q, Wang K, He Q, Wang Y and Hu F (2014). Antitumor activity of Chinese propolis in human breast cancer MCF-7 and MDA-MB-231 cells. *Evidence-Based Complementary and Alternative Medicine,* 2014**:**1-11.

Yagmurca M, Erdogan H, Iraz M, Songur A, Ucar M and Fadillioglu E (2004). Caffeic acid phenethyl ester as a protective agent against doxorubicin nephrotoxicity in rats. *Clinica Chimica Acta,* 348**:**27-34.

Yamaguchi M, Hamamoto R, Uchiyama S, Ishiyama K and Hashimoto K (2006). Anabolic effects of bee pollen Cistus ladaniferus extract on bone components in the femoral-diaphyseal and-metaphyseal tissues of rats in vitro and in vivo. *Journal of health science,* 52**:**43-49.

Yamaguchi M, Uchiyama S and Nakagawa T (2007). Anabolic effects of bee pollen Cistus ladaniferus extract in osteoblastic MC3T3-E1 cells in vitro. *Journal of health science,* 53**:**625-29.

Yasumoto R, Kawanishi H, Tsujino T, Tsujita M, Nishisaka N, Horii A and Kishimoto T (1995). Clinical evaluation of long-term treatment using cernitin pollen extract in patients with benign prostatic hyperplasia. *Clinical Therapeutics,* 17**:**82-87.

Yıldız O, Can Z, Saral Ö, Yuluğ E, Öztürk F, Aliyazıcıoglu R, Canpolat S and Kolaylı S (2013). Hepatoprotective potential of chestnut bee pollen on carbon tetrachloride-induced hepatic damages in rats. *Evidence-based complementary and alternative medicine,* 2013.

Zhang J, Cao X, Ping S, Wang K, Shi J, Zhang C, Zheng H and Hu F (2015). Comparisons of ethanol extracts of chinese propolis (poplar type) and poplar

gums based on the antioxidant activities and molecular mechanism. *Evidence-Based Complementary and Alternative Medicine*.

Other references from internet sources and official web portals:

European Commission DG Agriculture and Rural Development. Evaluation of measures for the apiculture sector. 2013; 1-170. Report No 30-CE-0219319/00-20.

Guidance for Industry: Proper labeling of honey and honey products by Center for Food Safety and Applied Nutrition. US Department of Health and Human Services. Food and Drug Administration. 2014.

"Honey". Report by National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA). 2014. ISSN: 1949-1492.

Official web portal of Agricultural and processed food products Export Development Authority (APEDA), Government of India.
http://apeda.gov.in/apedawebsite/SubHead_Products/Natural_Honey.htm
http://agriexchange.apeda.gov.in/product_profile/exp_f_india.aspx?categorycode=0408

Chapter 2. Scope and Objectives of Work:

This chapter discusses different approaches to be used in studies to meet the revealed need of work for bee pollen, propolis and CAPE. The specific objectives and study designs individually for bee pollen, propolis and CAPE have been listed herein.

2. SCOPE AND OBJECTIVES OF WORK:

A. Multicomponent systems:

I. BEE POLLEN: Exploration and Potentiation of Nutraceutical Competence

- Present study was designed with the aim to explore nutritional value, chemical composition and beneficial effects of Indian bee pollen in order to assess its candidature as a nutraceutical agent.
- The composition of bee pollen varies with plant source and geographic origin. Standard quality pollen with minimal variations can be obtained by collecting bee pollen from single botanical taxa, such pollen are termed as monofloral pollen. The monofloral pollen ensures uniform organoleptic and biochemical characteristics to that of original plant, while the heterofloral pollen exhibit variable properties (Almeida ML et al., 2005). Among various pollen yielding sources, Mustard crops (Family: Brassicaceae) are one of the major pollen yielding source in India. These crops have been extensively used in diet worldwide and possess economic significance (Cartea et al., 2010, Kumar and Andy, 2012). The phenolic composition of *Brassica* vegetables has been well established, however the nutritional and chemical composition of pollen from these sources are not yet explored. Therefore, in the present study monofloral Indian mustard bee pollen (MIMBP), i.e. *Brassica juncea* were selected for determination of nutritional value, chemical composition and beneficial effects on laboratory animals.
- Lipid based systems in the form of mixture of oil and surfactants have been reported to improve the solubility and bioavailability of poorly soluble actives (Pouton, 2000). Several lipid excipients comprising medium chain triglycerides (MCTs) have been documented for improving the poor solubility of phytoconstituents and thereby increasing their extraction efficiency (Aher et al., 2009, Sathiyanarayanan et al., 2010, Ketkar et al., 2011). Also, surfactants, on account of their amphiphilic nature comprising both hydrophobic and hydrophilic moieties, have been employed for extraction of several phytocompounds especially phenolics (Yazdi, 2011, Hosseinzadeh et al., 2013). Considering the ability of MCT oils and surfactants to improve solubility and extraction efficiency, present study was designed to

investigate applicability of edible lipid and surfactant combinations for processing of neat MIMBP to produce processed pollen (PMIMBP) with improved availability of polyphenols entrapped within the pollen shell.

- Skeletal muscles are highly sensitive to a variety of external stimuli, especially exercise. Heavy endurance training in the form of forced swimming exercise induces oxidative stress due to increased production of reactive oxygen or nitrogen species in skeletal muscle tissues (Thirumalai et al., 2011). To ascertain the beneficial role of bee pollen during physical activity, current work was designed to investigate effect of MIMBP and PMIMBP in chronic swimming exercise induced oxidative stress implications in skeletal muscle of Wistar rats.

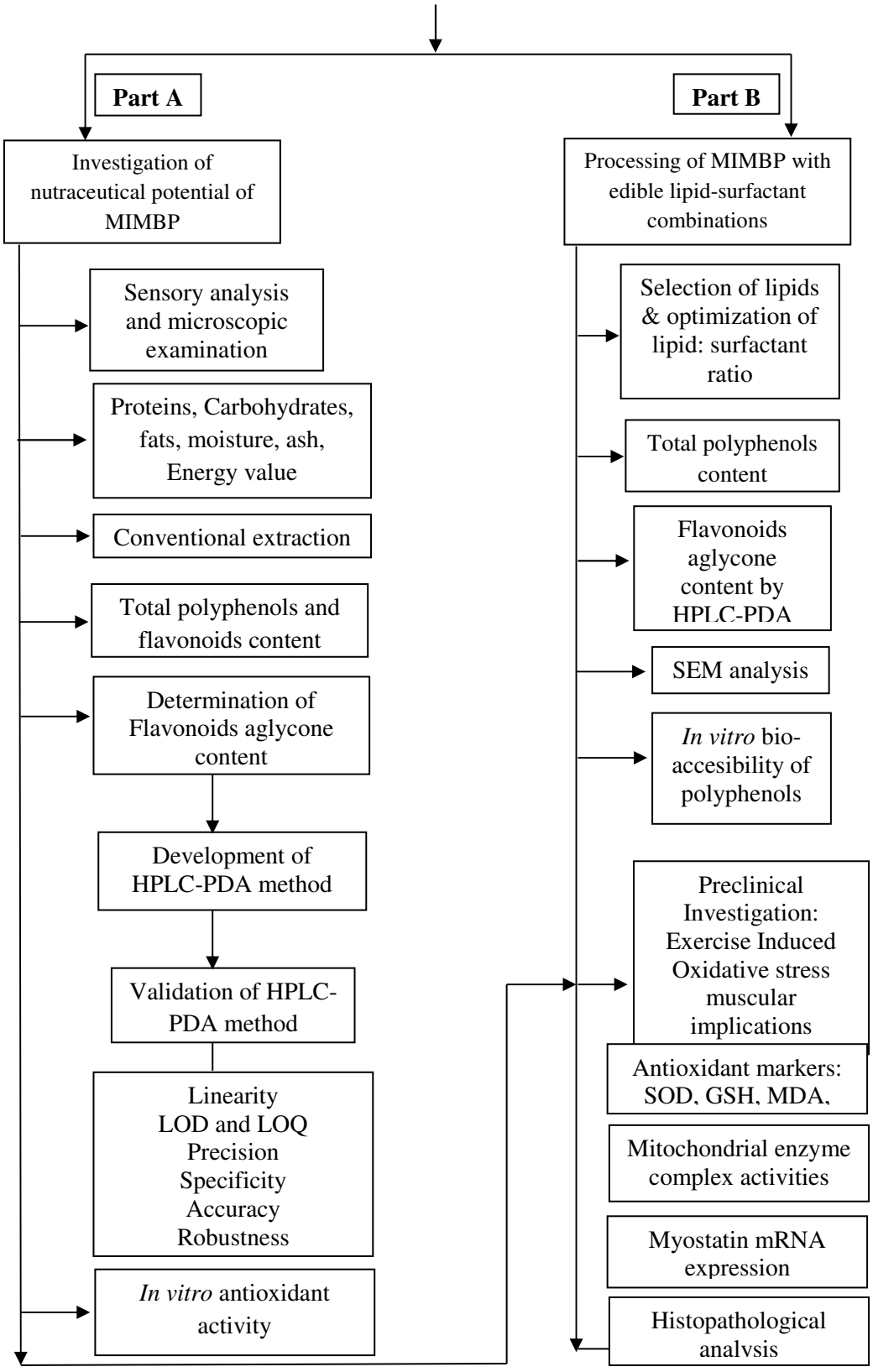
With this background the principal objectives of present study on bee pollen are as follows:

i) To investigate nutraceutical potential of monofloral Indian mustard bee pollen (MIMBP) in terms of nutritional value and chemical composition

ii) To develop a processing method for MIMBP using pharmaceutically acceptable excipients (edible lipid : surfactant mixtures) to produce processed pollen (PMIMBP) with improved availability of polyphenols and;

iii) To investigate the influence of neat and processed bee pollen supplementation on chronic swimming exercise induced oxidative stress implications in the gastrocnemius muscle of Wistar rats.

Figure 2.1. Study Design:
Bee Pollen: ***Exploration and Potentiation of Nutraceutical Competence***



II. PROPOLIS: Exploration of Green Extraction Method

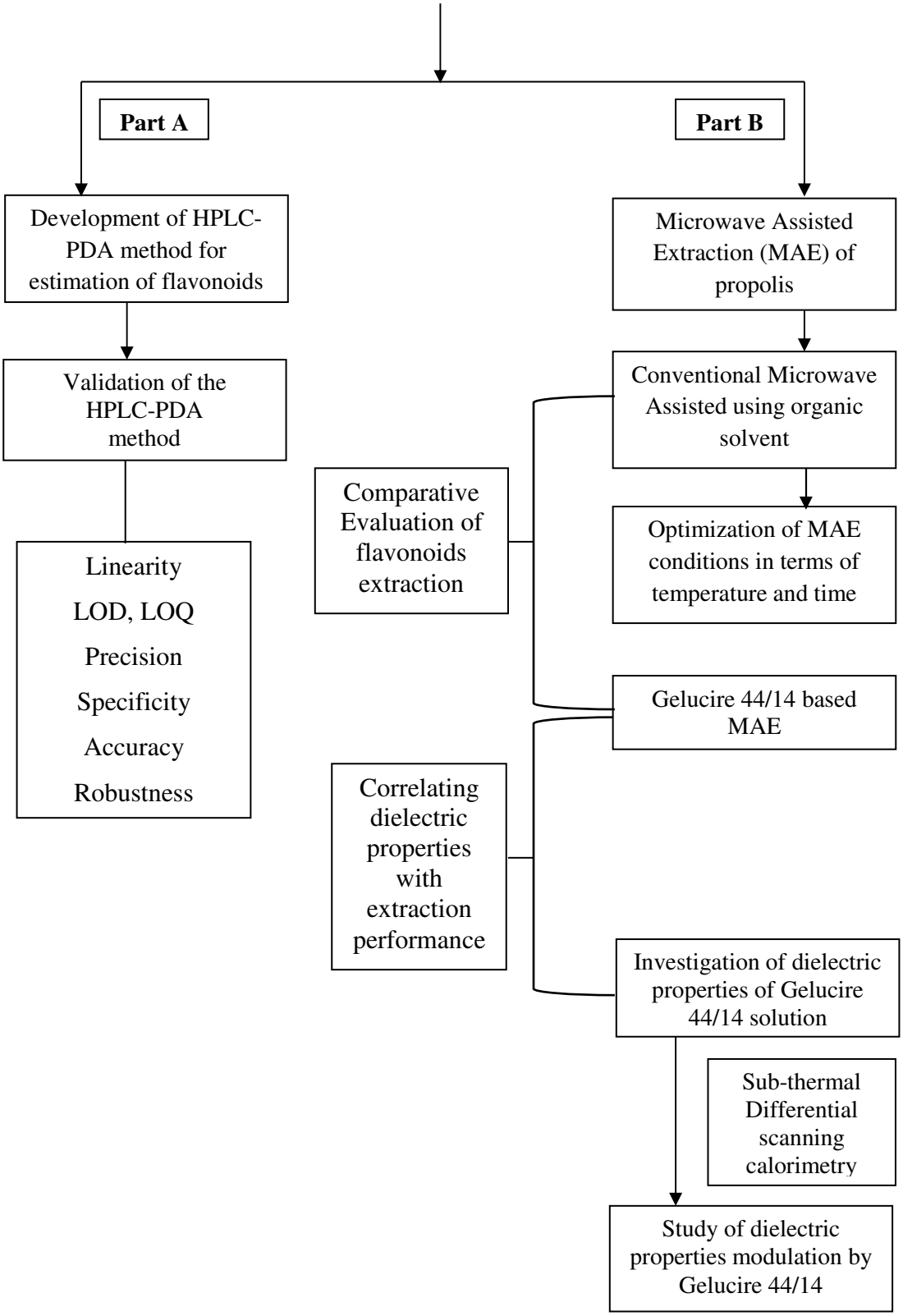
For fortification of propolis, present study was designed to improve extraction of bioactive flavonoids from propolis without use of conventional organic solvents.

- Microwave assisted extraction 'MAE' has been documented to be a better alternative to traditional methods for the extraction of phenolics, flavonoids and other non-phenolic actives from raw propolis (Trusheva et al., 2007, Jang et al., 2009, Moret et al., 2010, Pellati et al., 2013). The green technique MAE offers several merits in terms of shorter extraction time, higher extraction yield, less solvent consumption and low cost involved as compared to conventional extraction methods.
- Based on these facts and considering the need of green method for extraction of propolis, present work attempts to explore applicability of Gelucire 44/14 as an extractant in MAE of propolis, thereby avoiding the use of organic solvents. Gelucire 44/14 is an inert hydrophilic lipid documented to improve solubility, dissolution rate, membrane permeability, intestinal absorption and bioavailability of poorly water-soluble constituents (Damian et al., 2000, Koga et al., 2002); (Chambin and Jannin, 2005, Barker et al., 2003). Utilizing these properties, our group has established efficient method for extraction for herbals using Gelucire 44/14 as solvent (Aher et al., 2009, Lohidasan et al., 2009, Ketkar et al., 2011, Bothiraja et al., 2012).
- Accordingly, present study investigates suitability of Gelucire 44/14 in MAE process by determining the extraction yield of propolis flavonoids. Further attempts were made to explore the mechanism behind Gelucire 44/14 based MAE process in view of dielectric properties of the Gelucire 44/14 solutions used.

The principal objectives of present study on propolis are as follows:

i. To develop green extraction method for producing fortified and formulation friendly extract of propolis
ii. To investigate applicability of Gelucire 44/14 as an extractant in MAE of propolis and
iii. To investigate the dielectric properties of Gelucire 44/14 solutions and correlate with the extraction performance.

Figure 2.2. Study Design: Propolis: Exploration of Green Extraction Method



B. Single component system:

Caffeic Acid Phenethyl Ester (CAPE):

Present study was designed to study and modulate the physicochemical properties of CAPE in view of improving its aqueous solubility.

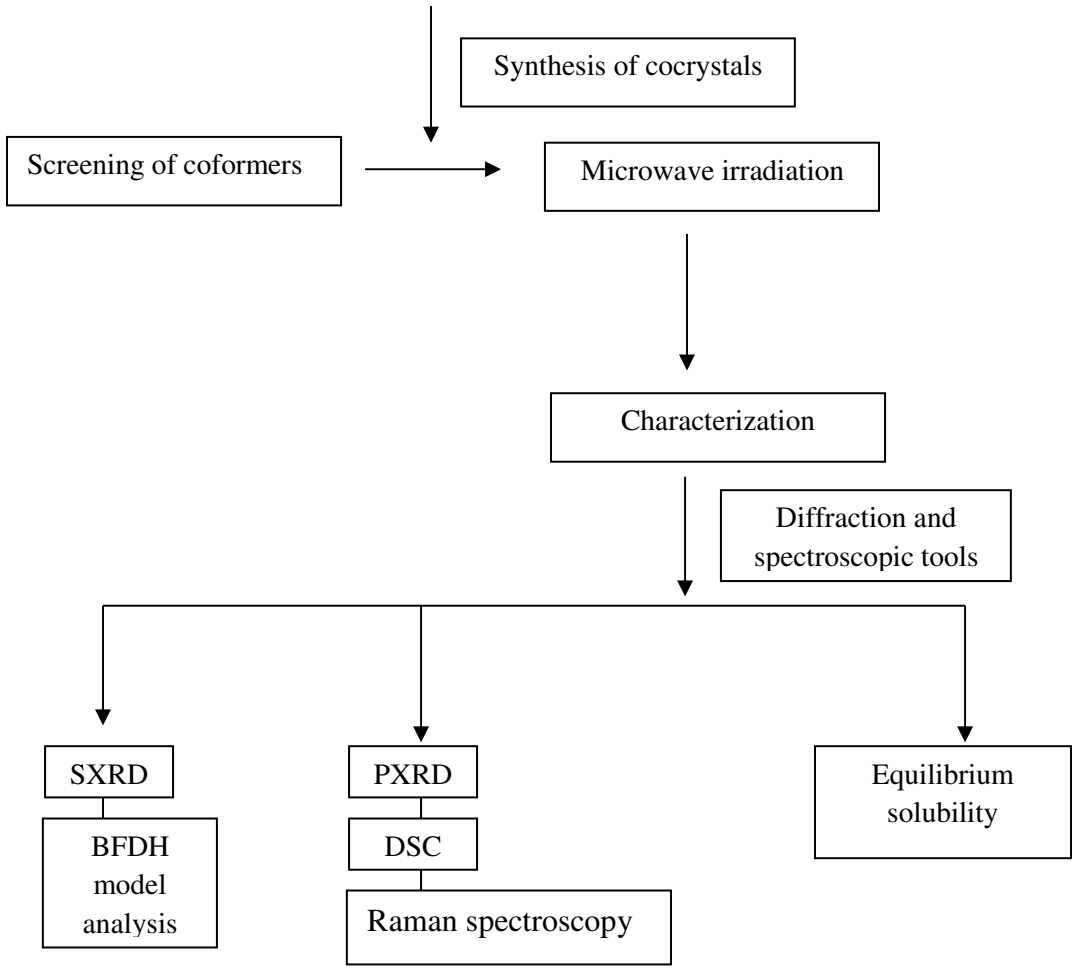
- "Cocrystallization", a process by which an active drug molecule and a coformer are brought together in the same crystal lattice (in definite stoichiometry), is now an established method for preparation of novel solid forms of active drug molecule with tailored physicochemical properties. 'Co-crystals' have received major attention of research worldwide, aiming to improve solubility; bioavailability; physicochemical stability; compressibility and therapeutic efficiency of active pharmaceutical ingredients (Jones et al., 2006, Shan and Zaworotko, 2008).
- Several scientific reports documents that cocrystallization has been successfully applied to modulate the solubility of various polyphenolics. For instance, the solubility and dissolution rate of curcumin, myricetin were improved by cocrystallizing with suitable co-formers (Sanphui et al., 2011, Sowa et al., 2014, Hong et al., 2015). Further, cocrystallization was documented to be advantageous in altering the pharmacokinetic profile of quercetin and epigallocatechin-3-gallate (Smith et al., 2011, Smith et al., 2013).
- Based on these facts, the present study was undertaken to develop cocrystals of CAPE using pharmaceutically acceptable coformers with an aim to improve the aqueous solubility of CAPE. Further attempts were made to understand the crystal structure landscape of CAPE directed towards improving its physicochemical properties with special reference to solubility.
- Recently, microwave assisted co-crystallization has been explored as a green technique for co-crystallization of active pharmaceutical ingredients. It employs use of microwaves as a source of energy for rapid crystallization and requires minimal amount of solvents (Pagire et al., 2013). Therefore attempts were made to develop CAPE-cocrystals by microwave assisted cocrystallization technique.

The principal objectives of present study on CAPE are as follows:

1. To develop cocrystals for CAPE using pharmaceutically acceptable GRAS coformers

2. To study physicochemical properties of CAPE and CAPE-cocrystals with special reference to aqueous solubility.

Figure 2.3. Study Design:



References:

Aher S, Biradar S, Gopu C and Paradkar A (2009). Novel pepper extract for enhanced P-glycoprotein inhibition. *Journal of Pharmacy and Pharmacology,* 61**:**1179-86.

Almeida ML, Pamplona LC, Coimbra SL and Barth OM (2005). Chemical composition and botanical evaluation of dried bee pollen pellets. *Journal of food composition and analysis,* 18**:**105-11.

Barker S, Yap S, Yuen K, Mccoy C, Murphy J and Craig D (2003). An investigation into the structure and bioavailability of α-tocopherol dispersions in Gelucire 44/14. *Journal of controlled release,* 91**:**477-88.

Bothiraja C, Pawar AP, Dama GY, Joshi PP and Shaikh KS (2012). Novel solvent-free gelucire extract of Plumbago zeylanica using non-everted rat intestinal sac method for improved therapeutic efficacy of plumbagin. *Journal of pharmacological and toxicological methods,* 66**:**35-42.

Cartea ME, Francisco M, Soengas P and Velasco P (2010). Phenolic compounds in Brassica vegetables. *Molecules,* 16**:**251-80.

Chambin O and Jannin V (2005). Interest of multifunctional lipid excipients: case of Gelucire® 44/14. *Drug development and industrial pharmacy,* 31**:**527-34.

Damian F, Blaton N, Naesens L, Balzarini J, Kinget R, Augustijns P and Van Den Mooter G (2000). Physicochemical characterization of solid dispersions of the antiviral agent UC-781 with polyethylene glycol 6000 and Gelucire 44/14. *European journal of pharmaceutical sciences,* 10**:**311-22.

Hong C, Xie Y, Yao Y, Li G, Yuan X and Shen H (2015). A Novel Strategy for Pharmaceutical Cocrystal Generation Without Knowledge of Stoichiometric Ratio: Myricetin Cocrystals and a Ternary Phase Diagram. *Pharmaceutical research,* 32**:**47-60.

Hosseinzadeh R, Khorsandi K and Hemmaty S (2013). Study of the effect of surfactants on extraction and determination of polyphenolic compounds and antioxidant capacity of fruits extracts.

Jang MJ, Sheu SR, Wang CC, Yeh YL and Sung KH 2009. Optimization analysis of the experimental parameters on the extraction process of propolis. *Proceedings of the International MultiConference of Engineers and Computer Scientists.* Citeseer.

Jones W, Motherwell W and Trask AV (2006). Pharmaceutical cocrystals: an emerging approach to physical property enhancement. *MRS bulletin,* 31**:**875-79.

Ketkar S, Gopu C, Badgujar L, Paradkar A and Mahadik K (2011). Male sexual behavior improving effect of lipid based extract of Mucuna Pruriens in rats. *Pharmacologyonline,* 1**:**1-14.

Koga K, Kawashima S and Murakami M (2002). In vitro and in situ evidence for the contribution of Labrasol® and Gelucire 44/14 on transport of cephalexin and cefoperazone by rat intestine. *European journal of pharmaceutics and biopharmaceutics,* 54**:**311-18.

Kumar S and Andy A (2012). Health promoting bioactive phytochemicals from Brassica. *Int Food Res J,* 19**:**141-52.

Lohidasan S, Paradkar AR and Mahadik KR (2009). Nootropic activity of lipid-based extract of Bacopa monniera Linn. compared with traditional preparation and extracts. *Journal of Pharmacy and Pharmacology,* 61**:**1537-44.

Moret S, Purcaro G and Conte LS (2010). Polycyclic aromatic hydrocarbons (PAHs) levels in propolis and propolis-based dietary supplements from the Italian market. *Food chemistry,* 122**:**333-38.

Pagire S, Korde S, Ambardekar R, Deshmukh S, Dash RC, Dhumal R and Paradkar A (2013). Microwave assisted synthesis of caffeine/maleic acid co-crystals: the role of the dielectric and physicochemical properties of the solvent. *CrystEngComm,* 15**:**3705-10.

Pellati F, Prencipe FP, Bertelli D and Benvenuti S (2013). An efficient chemical analysis of phenolic acids and flavonoids in raw propolis by microwave-assisted extraction combined with high-performance liquid chromatography using the fused-core technology. *Journal of pharmaceutical and biomedical analysis,* 81**:**126-32.

Pouton CW (2000). Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying'drug delivery systems. *European Journal of Pharmaceutical Sciences,* 11**:**S93-S98.

Sanphui P, Goud NR, Khandavilli UR and Nangia A (2011). Fast dissolving curcumin cocrystals. *Crystal Growth and Design,* 11**:**4135-45.

Sathiyanarayanan L, Paradkar AR and Mahadik KR (2010). In vivo and in vitro antioxidant activity of lipid based extract of Bacopa monniera Linn. compared to conventional extract and traditional preparation. *European Journal of Integrative Medicine,* 2**:**93-101.

Shan N and Zaworotko MJ (2008). The role of cocrystals in pharmaceutical science. *Drug discovery today,* 13**:**440-46.

Smith AJ, Kavuru P, Arora KK, Kesani S, Tan J, Zaworotko MJ and Shytle RD (2013). Crystal engineering of green tea epigallocatechin-3-gallate (EGCg) cocrystals and pharmacokinetic modulation in rats. *Molecular pharmaceutics,* 10**:**2948-61.

Smith AJ, Kavuru P, Wojtas L, Zaworotko MJ and Shytle RD (2011). Cocrystals of quercetin with improved solubility and oral bioavailability. *Molecular pharmaceutics,* 8**:**1867-76.

Sowa M, Ślepokura K and Matczak-Jon E (2014). A 1: 1 pharmaceutical cocrystal of myricetin in combination with uncommon piracetam conformer: X-ray single crystal analysis and mechanochemical synthesis. *Journal of Molecular Structure,* 1058**:**114-21.

Thirumalai T, Therasa SV, Elumalai E and David E (2011). Intense and exhaustive exercise induce oxidative stress in skeletal muscle. *Asian Pacific Journal of Tropical Disease,* 1**:**63-66.

Trusheva B, Trunkova D and Bankova V (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry central journal,* 1**:**13.

Yazdi AS (2011). Surfactant-based extraction methods. *TrAC Trends in Analytical Chemistry,* 30**:**918-29.

Chapter 3. Materials and Instruments:

This chapter provides brief information about all the materials, instruments, softwares used during work.

3. MATERIALS AND INSTRUMENTS

MONOFLORAL INDIAN MUSTARD BEE POLLEN (MIMBP):

The MIMBP pellets were collected from 24 Parganas district of West Bengal, India (22°.35' N, 88°.44'E). The collected fresh pollen pellets were hand sorted by appearance to avoid possible contamination of pollen from other source. The pollen samples were identified and authenticated by Central Bee Research and Training Institute, Pune, India. [Voucher Specimen No (1/WB/2012)]. The fresh pollen were dried at temperature below 40°C and stored in freezer at about -15°C throughout study. The stored samples were observed with no signs of degradation or fermentation.

PROPOLIS:

Synonym: Bee glue

CAS No: 9009-62-5

Propolis comprises resinous material of waxy consistency collected by honeybees from vegetation sources. A de-waxed purified propolis was generously supplied by Natures Laboratory Ltd., Whitby, North Yorkshire, United Kingdom. The sample was stored in freezer at about -15°C throughout study. It has dark brown color and exhibits brittle and free flowing consistency at low temperatures while becomes sticky consistency at or above room temperature. The freezer stored samples were observed with no signs of degradation.

PHARMACEUTICAL EXCIPIENTS:

Captex 355	Gift sample from Abitech Corporations, Cleveland, USA.
Lauroglycol FCC	Gift sample from Gattefosse Inc, France
Labrafil M 1944	Gift sample from Gattefosse Inc, France
Tween 80	Purchased by Merck, Mumbai, India.
Gelucire 44/14	Gift sample from Gattefosse Inc, France

SOVLENTS, CHEMICALS AND REAGENTS:

Acetonitrile (HPLC grade):	Merck, Mumbai, India
Methanol (HPLC grade):	Merck, Mumbai, India

Petroleum ether (HPLC grade): Merck, Mumbai, India

Ethanol (AR grade):	Merck, Mumbai, India
Ethyl acetate (AR grade):	Merck, Mumbai, India
Sodium hydroxide:	Merck, Mumbai, India
Sodium carbonate:	Sigma-Aldrich, Bangalore, India
Sodium sulfate:	Sigma-Aldrich, Bangalore, India
Hydrochloric acid:	Merck, Mumbai, India
Sulfuric acid:	Merck, Mumbai, India
O-Phosphoric acid:	Merck, Mumbai, India
M-Phosphoric acid:	Merck, Mumbai, India
Diammonium sulfate:	Merck, Mumbai, India
Aluminum chloride:	Sigma-Aldrich, Bangalore, India
Mercuric oxide:	Sigma-Aldrich, Bangalore, India

Super oxide dismutase (SOD): Sigma-Aldrich, Bangalore, India

Malondialdehyde (MDA):	Sigma-Aldrich, Bangalore, India
Reduced glutathione (GSH):	Sigma-Aldrich, Bangalore, India
Thiobarbituric acid (TBA):	Hi-Media Laboratories Pvt. Ltd., Mumbai, India
MTT - tetrazolium salt:	Sigma-Aldrich, Bangalore, India
Foilin-Ciocalteu's phenol Reagent:	Merck, Mumbai, India
Quercetin:	Merck, Mumbai, India
Rutin:	Merck, Mumbai, India
Chrysin:	Merck, Mumbai, India
Kaempferol:	Merck, Mumbai, India
Apigenin:	Merck, Mumbai, India
Galangin:	Merck, Mumbai, India
Acacetin:	Merck, Mumbai, India
Gallic acid:	Sigma-Aldrich Company Ltd, Gillingham, UK
Caffeic acid phenethyl ester	Sigma-Aldrich Company Ltd, Gillingham, UK
Caffeine:	Sigma-Aldrich Company Ltd, Gillingham, UK
Nicotinamide:	Sigma-Aldrich Company Ltd, Gillingham, UK

Isonicotinamide:	Sigma-Aldrich Company Ltd, Gillingham, UK
p-coumaric acid:	Sigma-Aldrich Company Ltd, Gillingham, UK
Curcumin:	Sigma-Aldrich Company Ltd, Gillingham, UK
Isoniazide:	Sigma-Aldrich Company Ltd, Gillingham, UK
RT-PCR Kit:	Biotools B&M Labs, Spain
Primers:	
Myostatin & β-actin:	Synthesized & purchased from Amnion Biosciences, India.

INSTRUMENTS

Electronic balance:	Contech electronic balance, India
pH meter:	Toshniwal Process Instruments Ltd
Magnetic stirrer:	REMI Magnetic stirrer, India
Hot Air Oven:	Kumar Industries, India
Hot plate:	Meta-Lab Mumbai, India
Centrifuge Allegra^{TM} 64R:	Beckman Coulter, USA
Incubator:	Thermo Electron Corporation, USA

UV-Visible spectrophotometer: Jasco V-630, Japan

Jasco HPLC system with Jasco PU2089 Plus quaternary gradient pump and Jasco multi-wavelength detector (PDA): Jasco, Tokyo, Japan

Waters e-2695 system integrated with a PDA 2998 detector, Waters system, USA.

Waters symmetry C18 column (4.6 × 250 mm, 5 µm): Waters system, USA.

Thermo Hypersil BDS C18 guard column (30 mm×4.6 mm, 5µm): Thermo scietific, USA

Thermo-Hypersil GOLD C18RP column (250mm×4.0 mm, 5µm): Thermo scietific, USA

HiQ-Sil™ HS C18 column (250 mm×4.0 mm, 5 µm): Kromatek, Japan

Nicon E800 Eclipse compound microscope, Japan

Scanning electron microscope (Inca X Sight Model No. 6650-M): Oxford Instruments.

Monowave focused closed-vessel Discover system 908010: CEM, Matthews INC, USA

Monowave 300 Reactor: Anton Paar, Gmbh, Austria

85070B open ended coaxial line probe & E5071C vector network analyzer: Agilent Technologies, Malaysia.

Differential scanning calorimeter: Mettler Toledo 821e DSC instrument equipped with an intracooler: Mettler Toledo, Switzerland and TA Instruments DSC 2000

PXRD: Bruker D8 advanced diffractometer,

SXRD: Xcalibur Gemini EOS CCD diffractometer (Oxford Diffraction, Yarnton, UK)

Raman microscope and Renishaw InVia Reflex benchtop spectrometer coupled with 683 nm stabilized diode (Renishaw Plc., UK)

SOFTWARES:

ChromPass software, Jasco, Japan

85070D dielectric probe kit software, Agilent Technologies, USA

Empower 3 chromatography data software, Waters, USA.

GraphPad Instat, USA

EXCIPIENT PROFILES:

1) Captex 355:

Synonyms: Glycerol Caprylate Caprate; Octanoic/Decanoic Acid Triglyceride- Medium Chain Triglyceride (MCT)

CAS Number: 65381-09-1, 73398-61-5

Form: Liquid

Specifications:

Specification	Limit
Appearance/ Form at 25 °C	Light Yellow/Clear Liquid
Odor	Neutral
Acid Value (mg KOH/g)	0.1 max
Saponification Value (mg KOH/g)	325 – 340
Iodine Value, Calculated (cg I2/g)	0.5 max.
Moisture, Karl Fischer (%)	0.1 max.
Hydroxyl Value (mg KOH/g)	10.0 max.
Specific Gravity @ 77 ºF (25 ºC)	0.92 - 0.96
Viscosity, cP @ 68 ºF (20 ºC)	25 – 33
Cloud Point	23 ºF or less

Typical Fatty Acid Distribution by GLC:

6:0 Caproic Acid…… 6 % max.

8:0 Caprylic Acid……50-75 %

10:0 Capric Acid…… 22-45 %

12:0 Lauric Acid……. 4 % max.

Oral LD50: **>** 36 ml/kg in rats

> 25 ml/kg in mice

Pharmaceutical and Nutritional Applications:

• Carrier (vehicle);

• Solubilizer

• Lubricant

• Emollient (topical formulations)

• Energy Source

• Viscosity Modifier

Shelf-Life: 3 years.

Storage: Store in a dry location at ambient temperature.

(MSDS data sheet, Abitec, an ABF Excipient Company; www.Abiteccorp.com)

2) Lauroglycol FCC:

Synonyms: Propylene glycol monolaurate (type I) EP/NF.

CAS Number: (27194-74-7; 142-55-2)

Form: Liquid

Description:

The product is manufactured from raw materials of vegetable and synthetic origins.

Specification	**Limit**
Appearance	Oily liquid
Odour	Faint
Specific gravity at 20°c (d20/4)	0.900 to 0.930
Acid value	<= 2.50 mgkoh/g
Saponification value	210 to 245 mgkoh/g
Iodine value	<= 1.0 gi2/100g
Peroxide value	<= 6.0 meqo2/kg
Alkaline impurities	<= 30 ppm naoh

Water content	<= 1.00 %
Total ashes content	<= 0.10 %
Heavy metals content (pb)	< 10 ppm
Arsenic content	< 3 ppm
Free propylene glycol content	<= 1.5 %
Monoesters content	45.0 to 70.0 %
Diesters content	30.0 to 55.0 %
Caprylic acid (c8)	<= 0.5 %
Capric acid (c10)	<= 2.0 %
Lauric acid (c12)	>= 95.0 %
Myristic acid (c14)	<= 3.0 %
Palmitic acid (c16)	<= 1.0 %

Solubilities AT 20°C (Eur. Ph.)

Ethanol 96°: Very soluble

Chloroform, Methylene chloride: Very soluble

n-Hexane: Very soluble

Water: Insoluble

Applications:

Oral drug delivery: Excipient used in formulations for solubility and bioavailability enhancement; Water insoluble surfactant for SELF (self-emulsifying lipidic formulation).

Oral/Dermal drug delivery: Solubilizer for actives.

Dermal drug delivery: W/O surfactant. It improves the stability of emulsions

(Reference: Technical data sheet, Specification No. 3219/5 Gattefosse, France)

3) Labrafil M 1944 CS:

Synonyms: Oleoyl macrogol-6 glycerides EP / Oleoyl polyoxyl-6 glycerides NF.

CAS Number: 69071-70-1 (or 68424-61-3 + 9004-96-0)

Description:

Specification	**Limit**
Appearance	Liquid at 40°c
Odor	Faint
Specific gravity at 20°c (d20/4)	0.935 to 0.955
Viscosity at 20°c	75 to 95 mpa.s
Acid value	<= 2.00 mgkoh/g
Saponification value	150 to 170 mgkoh/g

Iodine value	75 to 95 gi2/100g
Hydroxyl value	45 to 65 mgkoh/g
Peroxide value	<= 12.0 meqo2/k
Alkaline impurities	<= 80 ppm naoh
Water content	<= 0.50 %
Free glycerol content	<= 3.0 %
Total ashes content	<= 0.10 %
Heavy metals content (pb))	< 10 ppm
Palmitic acid (c16)	4.0 to 9.0 %
Stearic acid (c18)	<= 6.0 %
Oleic acid (c18:1)	58.0 to 80.0 %
Linoleic acid (c18:2)	15.0 to 35.0 %
Linolenic acid (c18:3)	<= 2.0 %
Arachidic acid (c20)	<= 2.0 %
Eicosenoic acid (c20:1)	<= 2.0 %
1.4-dioxane content	<= 10 ppm
Ethylene oxide content	< 1 ppm

Uses:

Oral drug delivery: Excipient used in formulations for solubility and bioavailability enhancement; Water dispersible surfactant for SELF (self-emulsifying lipidic formulation).

Oral/Dermal drug delivery: Solubilizer for actives.

Dermal drug delivery: W/O surfactant. It improves the stability of emulsions.

Shelf-Life: 3 years.

Storage: Store in a dry location at ambient temperature.

Solubilities AT 20°C (Eur. Ph.)

- Ethanol 96°: Insoluble
- Methylene chloride: Very soluble
- n-Hexane: Soluble
- Water: Dispersible
- Mineral oils: Very soluble

(Reference: Technical data sheet, Specification No. 3063/6 Gattefosse, France)

4) Tween 80®

Nonproprietary Names: Tween 80 polysorbate

Synonym: Polysorbate 80, PEG (80) sorbitan monooleate, polyoxyethylenesorbitan monooleate

Chemical names: Polyoxyethylene 20 sorbitan monooleate

Molecular Formula: $C_{64}H_{124}O_{26}$

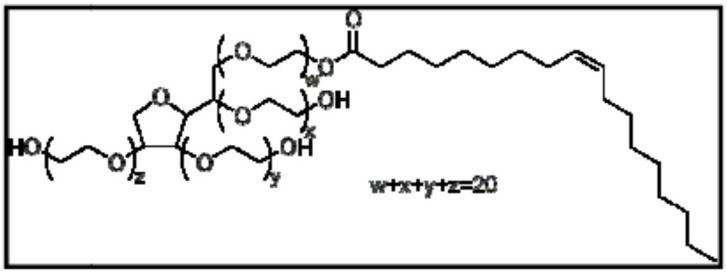
Molecular Weight: 1310 g/mol

Appearance: Yellow oily liquid

pH: 6-7

Flash point: 149 ˚C

Chemical structure:



HLB: 15

Relative density at 25˚C: 1.07 gm/cm^3

Viscosity (mPas): 425

Boiling point : >100 0C

Acute oral toxicity: LD 50 Rat 42.200mg/kg

Solubility: Very soluble in water, soluble in ethanol, cottonseed oil, corn oil, ethyl acetate, methanol, toluene

Functional Category: Dispersing agent; emulsifying agent; nonionic surfactant; solubilizing agent; suspending agent; wetting agent

Application: Polyoxyethylene sorbitan fatty acid esters (polysorbates) are a series of partial fatty acid esters of sorbitol and its anhydrides copolymerized with approximately 20, 5, or 4 moles of ethylene oxide for each mole of sorbitol and its anhydrides. The resulting product is therefore a mixture of molecules of varying sizes rather than a single uniform compound. Polysorbates containing 20 units of oxyethylene are hydrophilic nonionic surfactants that are used widely as emulsifying agents in the preparation of stable oil-in-water pharmaceutical emulsions. They may also be used as solubilizing

agents for a variety of substances including essential oils and oil-soluble vitamins, and as wetting agents in the formulation of oral and parenteral suspensions. They have been found to be useful in improving the oral bioavailability of drug molecules that are substrates for p-glycoprotein. The possible applications of Tween 80 are listed below:

Emulsifying agent

Used alone in oil-in-water emulsions 1-15 %

Used in combination with hydrophilic emulsifiers in oil-in-water emulsions 1 -10 %

Used to increase the water-holding properties of ointments 1- 10 %

Solubilizing agent

For poorly soluble active constituents in lipophilic bases 1-10 %

Wetting agent

For insoluble active constituents in lipophilic bases 0.1 -3 %

Stability and Storage Conditions: Polysorbates should be stored in a well-closed container, protected from light, in a cool, dry place.

Incompatibility: Discoloration and/or precipitation occur with various substances, especially phenols, tannins,tars, and tarlike materials. The antimicrobial activity of paraben preservatives is reduced in the presence of polysorbates.

(Reference: Handbook of pharmaceutical excipients, 6th edition, 2009, edited by Raymond C Rowe, Paul J Sheskey, Mariann E Quinn, Published by the Pharmaceutical Press and the Americal Pharmacists Association)

5) Gelucire 44/14

Synonyms: Lauroyl macrogol-32 glycerides EP, Lauroyl polyoxyl-32 glycerides NF, Lauroyl polyoxylglycerides (USA FDA IIG)

CAS Number: 57107-95-6

Form: Solid

Colour: Whitish

Odour: Light

General description: A non-ionic water dispersible surfactant composed of well characterized PEG-esters, a small glyceride fraction and free PEG. Able to self-emulsify on contact with aqueous media forming a fine dispersion ie. Microemulsion (SMEDDS).

Solubilizer and wetting agent: surfactive power improves the solubility and wettability of active pharmaceutical ingredients in vitro and in vivo. Bioavailability enhancer: increased bioavailability could be associated with inhibition of the enterocytic efflux transporter (known as P-gp inhibition) and of the of the enterocytic drug metabolizing enzyme CYP3A4. Good thermoplasticity for use as a binder in melt processes, associated with rapid formation of stable crystalline phase.

Gattefosse provides the following specifications:

Table 6.1: Specifications of Gelucire 44/14	
Colour	White
Odour	Faint
Melting point	42.5-47.5 ºC
Hydrophilic lipophilic balance (HLB)	14
Acid value (mgKOH/g)	< 2.00
Saponification value (mgKOH/g)	79-93
Iodine value (g I_2/100g)	< 2.0
Hydroxyl value (mgKOH/g)	36-56
Alkaline impurities (ppm NaOH)	< 80
Water content (%)	< 0.50
Free glycerol content (%)	< 3.00
Caprylic acid (C8)	<<12 %
Capric acid (C10)	15 %
Lauric acid (C12)	30 to 50 %
Myristic acid (C14)	5 to 25 %
Palmitic acid (C16)	4 to 25 %
Stearic acid (C18)	5 to 35 %

Physical Form: Semi-solid block

Hydrophilic-Lipophilic Balance (HLB): 14

Field of use: Human pharmaceutical products, veterinary products excluding food producing animals (EU).

Administration Route: Oral

Formulation techniques and dosage forms: Suitable for use in melt processing techniques: melt granulation (thermoplastic pelletization) and melt extrusion techniques for capsule filling, tabletting, sachets etc. Suitable for hard gelatin capsule molding. Suitable for adsorption onto neutral carrier powders for use in tablets, capsule filling and sachets.

Storage: Special temperature storage conditions are not required

Reference: http://www.gattefosse.com/en/applications/gelucire-4414.html

DRUG PROFILE:

Caffeic Acid Phenethyl Ester (CAPE):

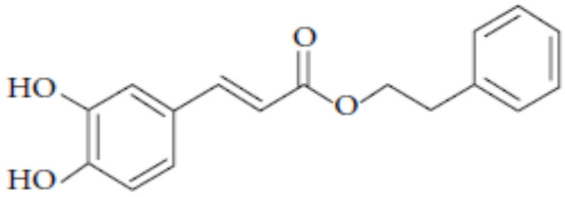
CAS number: 104594-70-9

Chemical name: Phenethyl 3,4-dihydroxycinnamate

Molecular formula: $C_{17}H_{16}O_4$

Molecular weight: 284.31 g/mol

Structural formula:



Description: white crystalline powder

Melting point: 129 ˚C

Storage: Store at −20°C . Avoid direct sunlight.

Solubility: Soluble in ethyl acetate (50 mg/mL), DMSO, ethanol, methanol, acetone, practically insoluble in water

Precautions: Keep away from skin and eyes. On exposure causes severe skin and eye irritation.

(Reference: MSDS data sheet, Sigma Aldrich)

Chapter 4. BEE POLLEN: *Exploration and Potentiation of Nutraceutical Competence*

This chapter will cover studies on monofloral Indian mustard bee pollen (MIMBP). Part A of the chapter includes nutritional and chemical analysis of MIMBP while Part B comprises studies on processing of MIMBP with edible lipid surfactant mixtures and their characterization. This will be followed by preclinical studies investigating effects of neat and processed bee pollen on chronic swimming exercise induced oxidative stress muscular implications in Wistar rats.

4A. Investigation of nutraceutical potential of monofloral Indian mustard bee pollen (MIMBP)

4A.1. Experimental Work:

4A.1.1. Sensory analysis and microscopic examination

The MIMBP was subjected to sensory analysis in terms of color, appearance, odor and taste. The pollen was subjected to standard acetolysis method (Erdtman, 1960) followed by microscopic examination using Nicon E800 Eclipse compound microscope in phase contrast mode with Image-ProPlus software. Measures of polar axis (P), equatorial diameter (E), colpus length (CL) and exine thickness were determined for 30 pollen grains under the microscope (×40). The pollen sample was observed under a scanning electron microscope, Oxford Instruments, Inca X Sight Model No. 6650-M. The sample was scattered on a 12 mm carbon grid attached to scanning electron microscope specimen mounts and were sputter coated with a layer of gold/palladium.

4A.1.2. Determination of nutrient composition:

4A.1.2.1. Moisture content

The moisture content of MIMBP was determined by repeated drying of sample in an oven at about 105 ℃ until constant weight was obtained (Escuredo et al., 2013).

4A.1.2.2. Total protein content

The total protein content was determined by Kjeldahl's method (Escuredo et al., 2013, Official methods of analysis of AOAC International, 2006) wherein MIMBP (2000 mg) was subjected to digestion by heating with a mixture of sodium sulfate (5000 mg) and mercuric oxide (300 mg) in concentrated sulfuric acid (25 ml) for about 6 h. The diluted sample solution was distilled with 0.1% sulfuric acid (50 ml) followed by addition of sodium sulfate solution (8%, 13 ml) and sodium hydroxide solution (40%, 50 ml). The ammonia collected (150 ml) was titrated with hydrochloric acid (0.1 mol/l). Protein content was estimated by multiplying the obtained percentage of nitrogen by a conversion factor of 5.6.

4A.1.2.3. Total fat content

Fat or lipid content of MIMBP (5000 mg) was determined by extracting with petroleum ether in a Soxhlet extractor at about 100 ℃/12 h (Official methods of analysis of AOAC International, 2005). The extraction flask was subjected to a heating and cooling cycle to evaporate the solvent completely followed by weighing of the mass. The difference in weight of the flask before and after extraction was correlated with fat content of the sample.

4A.1.2.4. Ash content

The ash content of MIMBP was determined by drying the sample at (550 ± 20) ℃ in a muffle furnace until constant weight (Mondal et al., 1998).

4A.1.2.5. Total carbohydrate content

Total carbohydrate content of MIMBP was determined by method based on calculating nutrient values from other components in the sample using the following formula (Schakel et al., 1997, Merrill and Watt, 1955).

Carbohydrates (g) = 100 – (protein (g) + fat (g) + moisture (g) + ash (g))

Total carbohydrate content of the sample estimated includes dietary fiber, as well as other components of the sample that are not lipid, protein, ash or water (Schakel et al., 1997).

4A.1.2.6. Energy value

The energy value for MIMBP was calculated based on Atwater numbers (Schakel et al., 1997, Merrill and Watt, 1955) using the following formula:

Energy Value (kcal) = 4 X (g protein + g carbohydrate) + 9 X (g fat)

4A.1.3. Determination of chemical composition

4A.1.3.1. Preparation of bee pollen extract

Finely ground MIMBP powder (1000 mg) was mixed with ethanol (99%; 20 ml), vortexed for 10 min and extracted at 70± 0.5℃ on a rotating mechanical shaker at 33987×g for 30 min (Carpes et al., 2007). The MIMBP extract obtained was

centrifuged at 33 987×g. The supernatant was filtered through a 0.45 μm membrane filter and subjected to determination of total polyphenol content and flavonoid content.

4A.1.3.2. Total polyphenol content

Total polyphenol content of MIMBP extract was determined by Folin-Ciocalteu colorimetric method (Singleton et al., 1999, Kumazawa et al., 2002, Marinova et al., 2005). Briefly, the extract (1 ml) was mixed with Folin-Ciocalteu's phenol reagent (1 ml). An aqueous solution of sodium carbonate (7%, 10 ml) was added to the mixture followed by dilution to 25 ml with distilled water. Absorbance was measured at 760 nm using JascoV-630 UV-Vis spectrometer after 90 min incubation of mixture at room temperature. The total polyphenol content was expressed in terms of mg gallic acid equivalent (GAE)/100 g of pollen. Calibration curve for gallic acid was performed in distilled water.

4A.1.3.3. Total flavonoid content

Total flavonoid content of MIMBP extract was determined by aluminum chloride colorimetric method (Kumazawa et al., 2002, Marinova et al., 2005, Woisky and Salatino, 1998). To MIMBP extract (1.5 ml), aluminum chloride ethanolic solution (1.5 ml, 2%) was added. The mixture was incubated for 1 h at room temperature and absorbance was measured at 420 nm. Total flavonoid content of the extract was expressed in terms of mg quercetin equivalent (QE)/100 g of pollen. Calibration curve for quercetin was performed in methanol.

4A.1.4. Determination of free flavonoid aglycones from bee pollen

4A.1.4.1. Preparation of standard solution

Standard stock solutions of rutin, chrysin, kaempferoland quercetin (1 mg/ml) were prepared in methanol. These were diluted with methanol to obtain mixed working standard solutions of concentration 10, 20, 40, 60, 80 and 100 μg/ml each.

4A.1.4.2.Preparation of MIMBP sample solution

Free flavonoid aglycones from MIMBP were determined using a process described by (Bonvehi et al., 2001) with minor modifications. Finely ground MIMBP powder

(2500mg) was mixed with ethyl acetate (25 ml). Diammonium sulfate (40%, 12.5 ml) and meta-phosphoric acid (20%, 2.5 ml) were added to the mixture, followed by shaking for 20 min. The extract was filtered through a 0.45 μm membrane filter under pressure. The filtrate was transferred into a separating funnel. The organic phase was collected and the extraction process was repeated. The organic phase collected was dried under reduced pressure at < 40 ℃. The residue was reconstituted in methanol (1.5 ml), filtered through a 0.45 μm nylon syringe filter and subjected to HPLC analysis for determination of free flavonoid aglycones.

4A.1.4.3. HPLC-PDA analysis

Instrumentation:

HPLC analyses were performed using Jasco HPLC system (Tokyo, Japan) on a Thermo Hypersil BDS C18 guard column (30 mm × 4.6 mm, 5 μm) coupled to a Thermo-Hypersil GOLD C18 RP column (250 mm × 4.0 mm, 5 μm) using Jasco PU2089Plus quaternary gradient pump, Jasco multiwavelength detector (PDA), ChromPass software and a Rheodyne injector with 20 μL loop. Elution was carried out with flow rate of 1 mL/min at ambient temperature. The solvents comprised water adjusted to pH 3.0 with ortho-phosphoric acid (solvent A) and methanol (solvent B) mixed using a linear gradient system; initial 30% B, 30%-50% B in 5 min, 50%-70% B in 10 min, 70%-75% B in 15 min followed by isocratic 75% B until 17 min. Solvent B was decreased to 30% over the next 3 min and held constant until the end of 25 min of run. Detection was performed between 200 to 400 nm and chromatograms were extracted at respective λmax of each flavonoid aglycone compound for improved sensitivity. The retention times (Rt) and UV spectra of flavonoid aglycones from the sample solution were compared with that of the standards and quantification was done using calibration curves of the standard solutions.

4A.1.4.4. Method validation

The developed HPLC-PDA method was validated based on following parameters as per ICH guidelines (Guideline ICH, 1996).

4A.1.4.4.1. Linearity and range:

Linearity of the method was studied by injecting the mixed working standard solutions of concentrations 10, 20, 40, 60, 80 and 100 µg/ml each in triplicate into the HPLC system. The peak area was recorded for individual flavonoid markers and calibration graph for each compound was obtained by plotting peak area versus concentration at each level.

4A.1.4.4.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is the smallest concentration of the analyte that gives a measurable response. LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified. The LOD and LOQ were determined for each compound by calibration curve method using standard deviation (Bernsdorff et al.) and slope (S) of the calibration curve for each compound as follows.

$$LOD = \frac{3.3 \times Sy.x}{S}; \quad LOQ = \frac{10.0 \times Sy.x}{S}$$

Where, Sy.x is Standard deviation of residuals from line; S is slope.

4A.1.4.4.3. Precision

The precision of system was ascertained by intra-day (repeatability) and inter-day (intermediate precision) injections at three different concentrations in triplicates. The repeatability studies were performed by injecting three different concentrations 10, 60 and 100 µg/ml of the mixed working standard solution three times on the same day and recording the corresponding peak areas. Intermediate precision studies were performed by analysis of the different concentrations 10, 60 and 100 µg/ml, three times on three different days. Relative standard deviation (% RSD) was determined to check the repeatability of sample application and measurement of peak areas.

4A.1.4.4.4.Specificity

Chromatograms of sample, standard and blank solutions were qualitatively compared and the peak purity values as detected by PDA detector for standards and sample solution were ascertained in order to ensure the specificity of method.

4A.1.4.4.5. Accuracy

Standard addition method was employed to determine percentage recovery of the flavonoid compounds from the MIMBP sample solution in triplicates. Prior to the addition of known quantities of standard markers, the background levels of individual flavonoids in extract sample were determined and used in the recovery experiment for determination of % recoveries.

4A.1.4.4.6. Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions in terms of flow rate, pH of mobile phase and analytical columns. Robustness of the method was ascertained at three different concentration levels viz. 10, 60 and 100 µg/ml for individual marker compounds.

4A.1.5. *In vitro* antioxidant activity of MIMBP

MIMBP were screened for in vitro antioxidant activity with respect to radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as per method described by (Campos et al., 2003, Almaraz et al., 2004, Leja et al., 2007). The dried MIMBP extract was reconstituted with ethanol (99%) to prepare different concentrations in the range of 10-100 µg/ml. The extract solution (1 ml) was mixed with methanolic solution of DPPH (5 mL; 0.1 mmol/l) and allowed to stand for 20 min at 27℃ in a dark place followed by centrifugation at 3056.1×g for 5 min. Reduction in DPPH radical concentration was analyzed by measuring the decrease in absorption at 517 nm detected by a UV visible spectrometer. DPPH-scavenging effect was calculated by the following formula:

$$\text{DPPH-scavenging effect (\%)} = (Ac - As/Ac) \times 100\%$$

Wherein Ac is absorbance of control solution; As is absorbance of the extract solution. The DPPH-scavenging effect of MIMBP extract was compared with that of the standard aqueous ascorbic acid solution.

4A2.Results and Discussion:

Sensory analysis and microscopic examination

Sensory analysis revealed that MIMBP were yellowish brown in color, and spherical to ovate in shape. They possessed a typical odor for pollen load with a sweet taste specific to Brassica pollen. The compound microscopic and SEM examination (Figure 4.1 A, B and C) revealed that the pollen exhibited subprolate to suboblate shape, medium trizonocolpate with ambtrilobed fossaperture confirming that these pollen belongs to the Indian mustard, i.e., *Brassica juncea*; Family: Brassicaceae. The size of individual pollen ranges between 25-30 µm (P: 30.54 µm×E: 25.06 µm). The CL of pollen grains was found to be 17.3-19.0 µm long, and0.25-0.32 µm wide. The exine was found to be about2.6 µm thick with reticulate ornamentation.

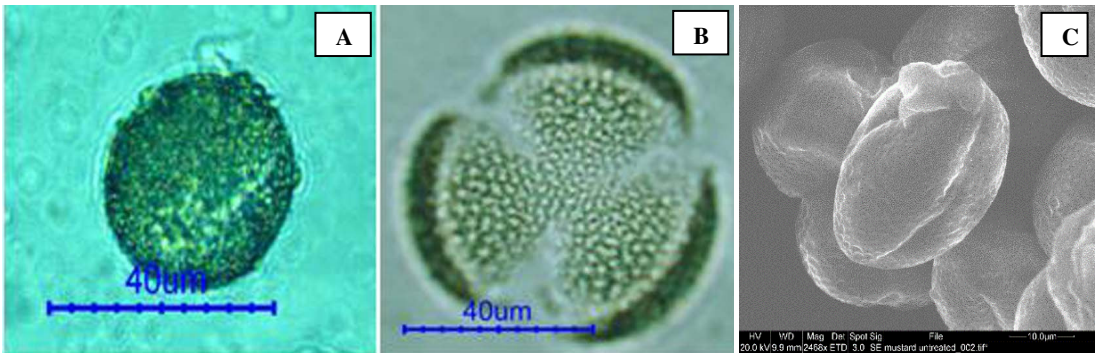


Figure 4.1. Microscopic images of MIMBP; A: Untreated pollen without acetolysis (40×);B: Acetolyzed pollen showing reticulate ornamentation (40×); C: Scanning electron microscope imageof MIMBP (154×)

Nutrient and chemical composition:

Observed values for nutritional and chemical composition of MIMBP are disclosed in table 4.1 as follows:

Table 4.1. Nutritional and chemical composition of MIMBP

Parameter	Content
A) Nutrient composition	
Total Proteins content	18.22 ±0.59 g/100g
Total fats content	13.77±0.68g/100g
Total carbohydrate content	56.06±1.74g/100g
Energy Value	421.09 ±1.61 Kcal/100g
Moisture content	7.260 ± 0.65 g/100g
Ash Content	2.567 ± 0.35 g/100g
B) Chemical composition	
Total Polyphenols content	1828.61 ±37.4 mg GAE/100g
Total Flavonoids content	122.35 ± 5.31mg QE/100g

Values are expressed as Mean ±SD (n=3)

Nutraceuticals have become targets of high commercial and research interests on account of their nutritional and therapeutic benefits (Rajasekaran et al., 2008). The current study explores nutraceutical candidature of MIMBP. The moisture content (7.260 ± 0.65 g/100g) observed for dried MIMBP falls within the acceptable range of proposed stipulations (Campos et al., 2008). Several countries have proposed minimal requirements for dried bee pollen including in Brazil: maximum 4% (w/w); Poland and Switzerland: maximum 6% (w/w); Uruguay: maximum 8% (w/w); Bulgaria: maximum 10%(w/w) (Campos et al., 2008). High protein content (18.22 ± 0.59 g/100g) observed with MIMBP is in agreement with the literature stating high protein content of Brassicaceae plants such as *Sinapis arvensis* and *Sinapis alba* (Szczesna, 2006b). MIMBP was found to have a high fat content (13.77 ± 0.68g/100g). The pollenkitt or pollen coat is known to be a major contributor to the fat content of bee pollen. The lipid fraction of MIMBP is one of the major sources of energy to bees, and plays a key role in the development, nutrition, and reproduction of bees (Roulston and Cane, 2000; Szczesna, 2006a). The high carbohydrate content (56.06 ± 1.74g/100g) in MIMBP makes it a rich source of sugars, contributing to its sweet taste and high caloric value (421.09 ±1.61 Kcal/100g).

Several reports have explored antioxidant potential of bee pollen in terms of radical-scavenging activity, total antioxidant activity, which have been well correlated

with total phenolic content of pollen samples (Campos et al., 1997, Feas et al., 2012, Campos et al., 2003, Almaraz et al., 2004, Leja et al., 2007, Marghitas et al., 2009). Equation for calibration curve of gallic acid in distilled water was found to be y = 0.152x + 0.006; with R^2 = 0.998. Further the equation for calibration curve of quercetin in methanol was found to be y = 0.0081x + 0.0612 with R^2 = 0.996. Results confirmed richness of MIMBP in antioxidant principles polyphenols (1828.61±37.4 mg GAE/100g) and flavonoids (122.35 ± 5.31mg QE/100g). Major polyphenol groups reported in Brassica species are flavonoids and phenolic acids, with the main flavonols being quercetin, kaempferol and isorhamnetin with hydroxycinnamic acids (Cartea et al., 2010). Flavonoids have been considered as principal indicating ingredient constituents of bee pollens (Campos et al., 1997).

HPLC method validation:

Developed HPLC-PDA method provides simple, specific and accurate analysis of most commonly observed flavonoid aglycones rutin, chrysin, kaempferol and quercetin from bee pollen. Linear gradient for mixing of the solvents (A i.e. water adjusted to pH 3.0 with ortho-phosphoric acid and B i.e. methanol) was optimized to achieve resolution of the screened analytes with stable baseline. Figure 4.2 depicts stacked view of representative chromatogram for the standard solution extracted at respective wavelengths of detection for each standard marker.

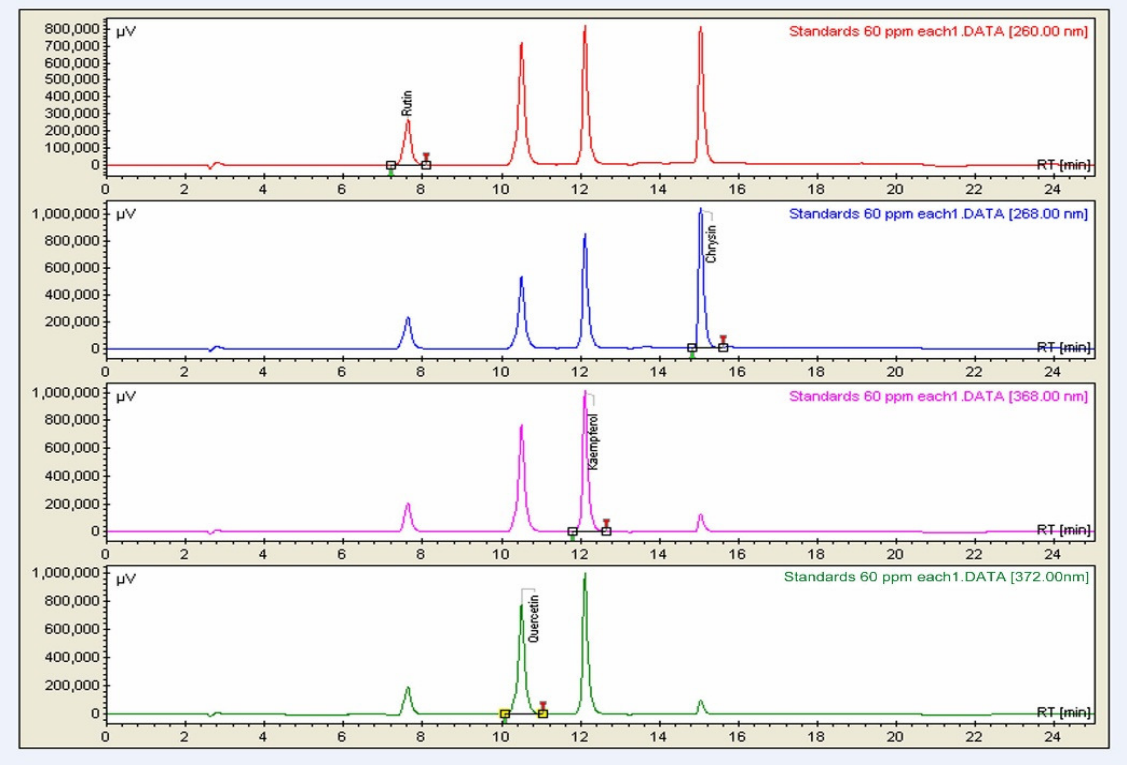


Figure 4.2. Stacked view of representative chromatogram of flavonoids standard solution

The chromatogram (PDA extracted at 260, 268, 368 and 372 nm for rutin, chrysin, kaempferol and quercetin respectively) illustrated well-resolved marker compounds within 25 minute run under gradient conditions. The Rt values (min ± SD) observed for standard markers are as follows: rutin (7.639 ± 0.020), chrysin (15.039 ± 0.010), kaempferol (12.106 ± 0.010) and quercetin (10.516 ± 0.010).

Figure 4.3 depicts UV spectra for the standards recorded with PDA multiwavelength detector.

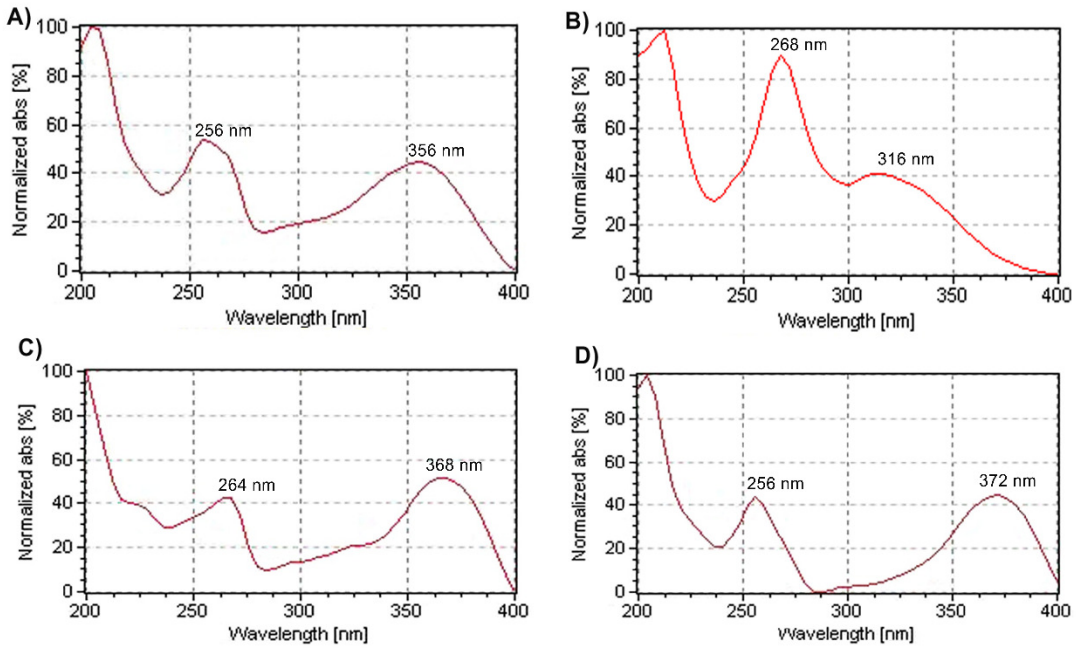


Figure 4.3. UV spectra for standards (A) rutin, (B) chrysin, (C) kaempferol, and (D) quercetin.

Results for validation parameters with ANOVA and residual analysis are summarized in Table 4.2.

Linearity:

The HPLC-PDA method for quantification of flavonoid markers was validated and showed good linearity (r^2> 0.998) in the concentration range of 10-100 µg/mL, wide enough to quantify constituents in the MIMBP sample. The calibration curve parameters for the screened markers showed linear relationship between peak area and concentration for each compound. Figure 4.4 A-D demonstrated linear calibration curves for individual marker compounds. Values of the slope and intercept for each compound are mentioned in table 4.2.

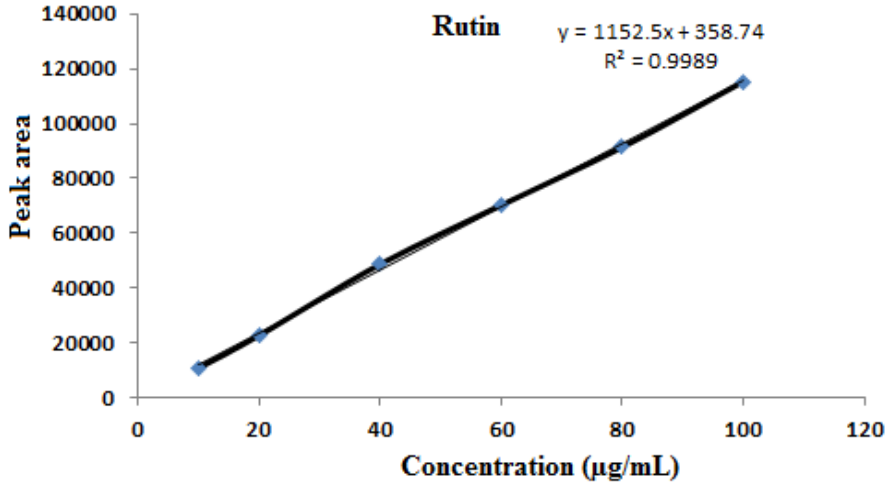


Figure 4.4A. Linear calibration curve of Rutin

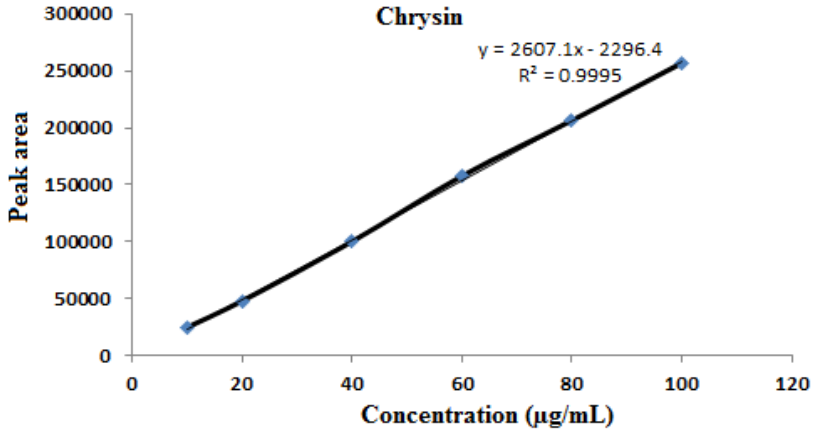


Figure 4.4B. Linear calibration curve of Chrysin

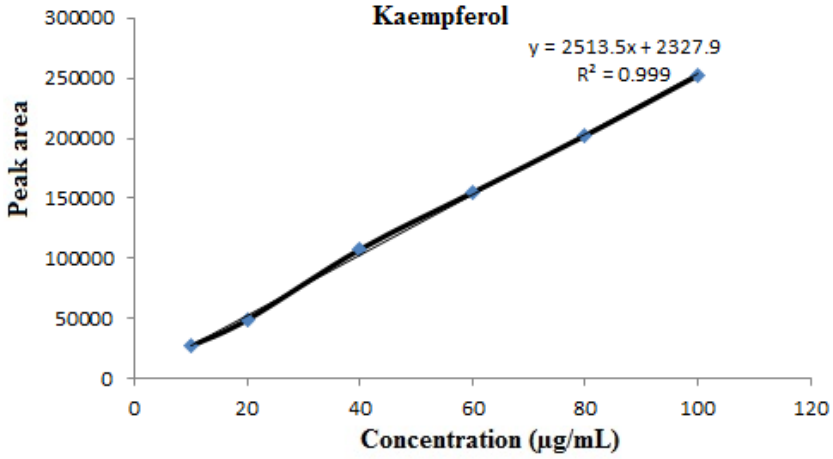


Figure 4.4C. Linear calibration curve of Kaempferol

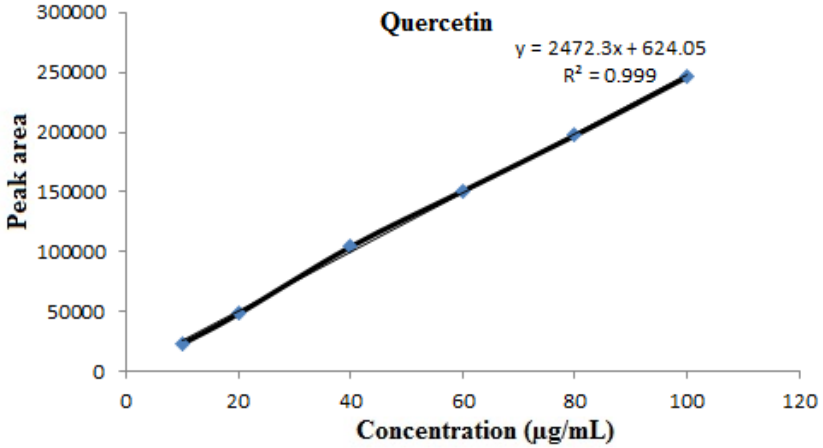


Figure 4.4D. Linear calibration curve of Quercetin

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ values obtained for the respective marker compounds are displayed in table 4.2. The values concluded that the developed method was sensitive.

Table 4.2. Summary of validation parameters with statistical data for calibration curves

Parameter	Rutin	Chrysin	Kaempferol	Quercetin
Linearity range (µg/mL)	10-100	10-100	10-100	10-100
Slope ± Standard error	1153 ± 18.76	2607 ± 30.44	2514 ± 40.30	2472 ± 39.25
Intercept ± Standard error	359.0 ± 1139	-2296 ± 1848	2328 ± 2446	624.4 ± 2382
Confidence limit of slope [a]	1100 to 1205	2523 to 2692	2402 to 2625	2363 to 2581
Confidence limit of intercept [a]	-2802 to 3520	-7425 to 2833	-4461 to 9117	-5988 to 7236
r^2	0.9989	0.9995	0.9990	0.9990
Sy.x [b]	1463	2374	3143	3061
P value [c]	<0.0001	<0.0001	<0.0001	<0.0001
Limit of detection (µg/mL)	1.20	0.58	0.33	0.62
Limit of quantitation (µg/mL)	3.64	1.76	0.99	1.88
Specificity	Specific	Specific	Specific	Specific
Mean Recovery %	NA	NA	100.06	98.52

(n=3); [a] 95% Confidence Intervals; [b]Standard deviation of residuals from line; [c]*P* value is <0.0001, considered extremely significant

Precision:

Table 4.2.1 displays % RSD values for all standards at respective concentrations of 10, 60 and 100 µg/ml. The % RSD values for all the standards were observed in the range of 0.136 to 2.091 indicating acceptable intra-day and inter-day variation with respect to working standards.

Table 4.2.1. Intra-day and inter-day precision data of HPLC-PDA method for flavonoid markers

Standard	Conc (µg/ml)	Repeatability (intra-day)			Intermediate precision (inter-day)		
		Found conc ± SD	% RSD	SE	Found conc ± SD	% RSD	SE
Rutin	10	9.810 ± 0.205	2.091	0.068	9.933 ±0.102	1.033	0.034
	60	59.403 ±1.21	2.041	0.404	59.373 ±1.071	1.803	0.357
	100	99.326 ±1.114	1.151	0.381	98.930 ±0.860	0.869	0.286
Chrysin	10	9.960 ±0.062	0.627	0.020	9.880 ±0.095	0.965	0.037
	60	59.923 ±0.162	0.271	0.054	59.883 ±0.100	0.167	0.033
	100	99.616 ±0.526	0.528	0.175	99.923 ±0.045	0.045	0.015
Kaempferol	10	9.763 ±0.111	1.142	0.037	9.896 ±0.020	0.210	0.006
	60	59.756 ±0.289	0.483	0.096	59.750 ±0.081	0.136	0.027
	100	99.633 ±0.540	0.542	0.180	99.520 ±0.314	0.316	0.104
Quercetin	10	9.863 ± 0.073	0.747	0.024	9.796 ±0.106	1.091	0.035
	60	59.766 ±0.250	0.418	0.083	59.530 ±0.595	1.001	0.198
	100	99.520 ±0.574	0.577	0.191	99.510 ±0.581	0.584	0.193

Specificity:

No peak interference for standards, sample and blank solutions at respective retention times as depicted in figures 4.2, 4.5 and 4.9 indicated specificity of method. Peak purity values for flavonoid peaks were greater than 999.877 for standard and sample solutions indicating absence of additional co-eluting peaks.

Accuracy:

The accuracy as measured by % recovery with small % RSD ranged from 98.10 % to 101.26% indicating reliability of method for quantification of screened flavonoids.

Table 4.2.2. Accuracy/recovery studies of HPLC method for flavonoids

Flavonoid	Added concentration (µg/ml)	Measured concentration (µg/ml)	% Recovery	% RSD
Kaempferol				
(14.25)	20	33.92	99.03	1.052
(14.46)	40	54.4	99.88	1.261
(14.32)	60	75.26	101.26	1.180
Quercetin				
(11.15)	20	30.56	98.10	0.961
(11.26)	40	50.43	98.38	1.884
(11.02)	60	70.37	99.08	1.912

Robustness:

The flow rate as a factor was varied at three levels (−1, 0 and +1) viz. 0.9, 1 and 1.1 ml/min. pH of mobile phase was varied as 2.5, 3 and 3.5. Two analytical columns viz, Hypersil GOLD C18 column (Thermo Scientific, USA) and HiQ-Sil™ HS C18 column (Kromatek, Japan) were employed during study. One factor at a time was changed to estimate the effect. The replicate injections (n = 3) of mixed standard solution at three concentration levels were performed under the changes of chromatographic parameters (factors). No marked changes in chromatograms were observed upon the deliberate changes made in parameters flow rate, pH of mobile phase and type of column indicating the robustness of developed method.

Determination of flavonoid aglycones from MIMBP:

Figure 4.5 depicts chromatogram for MIMBP sample solution with peaks at Rt 12.097 and 10.519 min resembling the standards kaempferol and quercetin, respectively.

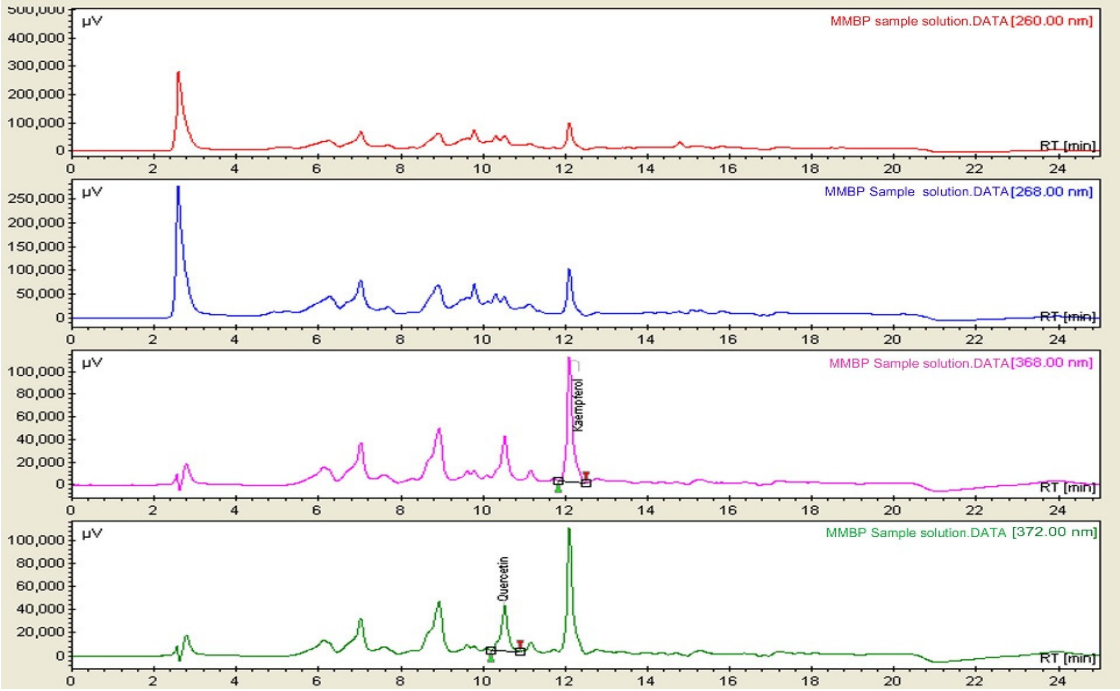


Figure 4.5. Stacked view of representative chromatogram of flavonoid aglycone compounds from the MIMBP sample solution

The retention times and UV spectra of flavonoid aglycones from MIMBP sample solution confirmed presence of kaempferol and quercetin in the MIMBP sample solution. Amongst the aglycones screened, kaempferol and quercetin were detected in amounts (6.54 ± 0.05 mg/100gm) and (5.14 ± 0.04 mg/100g) respectively.

The free flavonoid aglycone analysis has been considered an important parameter for defining the quality of bee pollen (Bonvehi et al., 2001, Tomas et al., 1989). Most pollen flavonoids exist in the form of glycosides, especially O-glycosides. Hydrolyzing the glycosides to aglycone provides a practical approach for effective determination of flavonoids from samples (Bonvehi et al., 2001). In case of honeybee-collected pollen apart from honey, hypopharyngeal gland secretions from the honeybee, along with the presence of hydrolyticenzymes α/β glucosidase, accompany the pollen pellets, which cause partial enzymatic hydrolysis of glycosides to free aglycone in the free state (Bonvehi et al., 2001). HPLC analysis confirmed the presence of flavonoids kaempferol and quercetin, which can be considered as markers for determining the quality of MIMBP.

In vitro antioxidant activity of MIMBP

Figure 4.6 demonstrates DPPH-scavenging effect of MIMBP extract and the standard ascorbic acid solution.

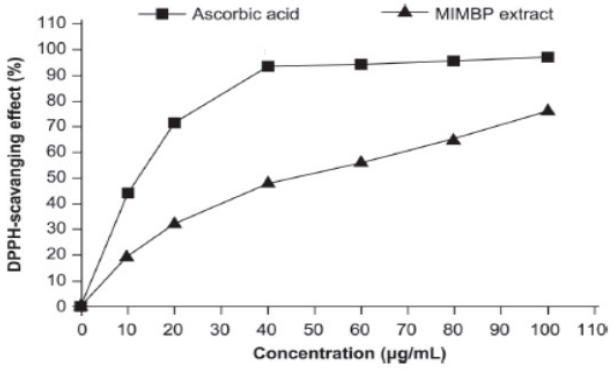


Figure 4.6. DPPH scavenging effect of MIMBP extract in comparison with ascorbic acid.

Both the MIMBP extract solution and the standard ascorbic acid solution exhibited a concentration-dependent increase in free DPPH radical scavenging effect as depicted in Figure 4.6. The standard ascorbic acid solution at 40 µg/ml showed more significant ($P<0.05$) scavenging effect on free DPPH radical activity as compared to that of the MIMBP extract solution. The IC_{50} values for the MIMBP extract and the standard ascorbic acid solution were found to be 54.79 and 18.13 µg/ml respectively. A significant reduction in DPPH concentration exhibited by MIMBP confirmed their antioxidant potential.

Nutraceuticals in the form of antioxidants, omega-3 polyunsaturated fatty acids, and certain vitamins are often recommended to prevent conditions associated with free radical damage, such as cardiovascular diseases, cancer, and diabetes (Rajasekaran et al., 2008). This suggests possible application of MIMBP in the prevention of such diseases.

4A.3. Conclusion

In conclusion, current study for the first time demonstrated nutraceutical candidature of MIMBP. Its rich nutritional value comprises more than about 50% by weight of carbohydrates, about 18% by weight of proteins, and over 10% by weight of fats which together makes MIMBP a high caloric source. This study also illustrated the chemical

composition of MIMBP in terms of polyphenol and flavonoid content, suggesting its potential use in the prevention of free radical-implicated diseases. The study further demonstrated development of simple, specific and accurate liquid chromatographic method for identification and quantification of free flavonoid aglycones, which can be applied for screening the quality of honeybee-collected pollen. The study outcome will be useful to set national pollen standards for monofloral bee pollen as an initial attempt in establishing quality criteria for bee pollen worldwide.

4B) POTENTIATION OF MIMBP: Processing with edible lipid surfactant combinations followed by preclinical investigations.

4B.1. Experimental work:

4B.1.1. Preliminary studies:

Ball milling:

The MIMBP (5000 mg) were subjected to milling using Retsch planetary Ball mill PM 100 CM for 15 min at revolution speed of 120 rpm and bead load 15 (medium). Upon completion of process, the milled pollen sample (1000 mg) was subjected to extraction as per section 4A.1.3.1 and determination of total polyphenols content as mentioned in 4A.1.3.2. Further the milled pollen sample was subjected to SEM analysis as described in 4A.1.1.

4B.1.2.Processing of pollen with an edible lipid-surfactant mixture:

4B.1.2.1. Selection of lipids:

i. Individually, MIMBP powder (1000 mg) was added to different pre-weighed mixtures of lipid and surfactant (500mg: 500mg) in mortar separately. The lipids screened for study include Captex 355, Lauroglycol FCC and Labrafil M 1944 with surfactant Tween 80.
ii. Individually the blends were vigorously triturated for a period of 10-15 min to ensure complete wetting and uniform mixing of MIMBP with the lipid-surfactant mixtures to produce processed pollen.
iii. For analysis, individual processed pollen masses were dispersed in distilled water (20 ml), vortexed for 10 min. The solutions were diluted with ethanol (99%, 10X) filtered through 0.45 µm membrane filter and subjected to determination of total polyphenols content as mentioned in 4A.1.3.2. The blank solutions were prepared using corresponding lipid-surfactant mixtures without pollen so as to nullify any possible interference of the lipid-surfactant mix. Final selection of the lipid-surfactant composition was made on the basis of observed highest amount of total polyphenols content for processed pollen sample.

4B.1.2.2. Optimization of lipid: surfactant ratio:

i. A fixed amount (1000 mg) of MIMBP was mixed with different weight (mg) ratios of Captex 355:Tween 80 (500:500, 500:750,750:500, and 750:750) separately in mortar.

ii. Individually the blends were vigorously triturated to ensure complete wetting and uniform mixing of MIMBP powder with the lipid-surfactant mixture to produce processed pollen.

iii. For analysis the pollen masses were subjected to determination of total polyphenol content as described above. The blank solutions were made by using corresponding amounts of Captex 355 and Tween 80 mixtures without pollen so as to nullify any possible interference of the lipid-surfactant mix. The lipid-surfactant ratio demonstrating highest extraction of total polyphenols was determined.

4B.1.3. Determination of free flavonoid aglycones from PMIMBP:

The processed pollen sample (PMIMBP; 2500 mg) demonstrating highest total polyphenols content was further subjected to determination of free flavonoid aglycone marker compounds as per the process described in 4A.1.4.2.

4B.1.4. Scanning electron microscopy:

The neat MIMBP and PMIMBP were subjected to standard acetolysis method (Erdtman, 1960) followed by to scanning electron microscopy (SEM) analysis as described in section 4A.1.1 using Oxford Instruments, Inca X Sight Model No. 6650-M. Around 30 pollen grains from both samples were studied.

4B.1.5. *In vitro* bio-accessibility of polyphenols from MIMBP and PMIMBP:

In vitro bio-accessibility of polyphenols from neat MIMBP and PMIMBP was investigated by subjecting them to *in vitro* gastric digestion protocol according to reported methods (Tagliazucchi et al., 2010, Bouayed et al., 2011) with little modifications. Individually, the neat MIMBP and PMIMBP (1000 mg each) were transferred to segments of dialysis bags previously soaked with 0.9% sodium chloride

solution. The sealed bags containing pollen samples were immersed into simulated gastric fluid (30 ml) containing sodium chloride (0.2 % w/v) and pepsin (300 U/ml) maintained at 37° ± 0.5 ˚C for 2 h at 100 rpm. The pH was adjusted to 1.2 with concentrated hydrochloric acid. Aliquots of samples were collected at different time intervals (5, 10, 15, 30, 45, 60 and 120 min) and subjected to determination of total polyhenols content as described in 4A.1.3.2. The analysis was done in triplicate. Calibration curve for gallic acid was performed in simulated gastric fluid.

4B.1.6. Preclinical investigation:

Effect of MIMBP and PMIMBP on exercise-induced oxidative stress muscular implications in Wistar rats

- Skeletal muscles are highly sensitive to a variety of external stimuli, especially exercise. Exhaustive exercise in the form of forced chronic swimming exercise induces oxidative stress in skeletal muscle tissues due to increased production of reactive oxygen or nitrogen species (Thirumalai et al., 2011). Increased oxidative stress triggers exercise-induced muscle injury on account of heavy eccentric contractions. The elevated intracellular reactive oxygen species (ROS) aggravates exercise-induced muscular weakness and fatigue (Smith and Lin, 2013).
- Furthermore, strenuous exercise may induce mitochondrial dysfunction which can cause oxidative damage and injury to tissues (Lima et al., 2013).
- Myostatin, a transforming growth factor-superfamily, plays a vital role in regulation of skeletal muscle mass. Rigorous exercise modulates myostatin mRNA expression in skeletal muscles affecting muscle mass (Matsakas et al., 2006).

Therefore, in order to combat the implications of oxidative stress during physical activity, many athletes and sportspeople need muscle protectant supplements. Present study investigates at biochemical, mitochondrial, and molecular level, effect of MIMBP and PMIMBP on exercise induced oxidative stress implications in gastrocnemius muscles of Wistar rats.

The experimental protocol (No. CPCSEA/40/12) was approved by the Institutional Animal Ethics Committee and performed according to the guidelines of

the Committee for Control and Supervision of Experimentation on Animals, Government of India.

4B.1.6.1.1.Acute oral toxicity test:

An acute oral toxicity test for MIMBP and PMIMBP was performed according to OECD guidelines number AOT 425. Accordingly, a total of 110 Swiss albino mice of either sex with an average body weight in the range of 28–30 g were randomly distributed into the vehicle control group, MIMBP treated, and PMIMBP treated groups. Five animals were assigned to each group. Each of the MIMBP and PMIMBP treated groups received oral doses of 55 mg/kg, 175 mg/kg, 550 mg/kg, 1750 mg/kg, and 2000 mg/kg, respectively for 14 days. The vehicle control group received gum acacia (10 mg/kg) per oral dose. The animals were continuously monitored for 42 hours to detect any changes in behavioral, respiratory or autonomic responses, restlessness, convulsions, tremors, salivation, diarrhea and mortality.

4B.1.6.1.2. Experimental protocol:

The Wistar rats were randomly divided into nine different groups with six rats in each group as follows:

(1) Non exercised groups

Group I: Normal: Rats were administered a single daily oral dose of vehicle gum acacia (10 mg/kg, for 4 weeks) and were not subjected to any chronic swimming exercise.

Group II: Per se: Rats were administered a single daily oral dose of PMIMBP (300 mg/kg, for 4 weeks) and were not subjected to any chronic swimming exercise.

(2) Exercised groups

Group III: Exercised control: Rats were administered a single daily oral dose of vehicle gum acacia (10 mg/kg, for 4 weeks) and subjected to chronic swimming exercise.

Group IV: MIMBP (100 mg/kg) + exercise: Rats were administered a single daily oral dose of MIMBP (100 mg/kg in gum acacia, for 4 weeks) and subjected to chronic swimming exercise.

Group V: MIMBP (200 mg/kg) + exercise: Rats were administered a single daily oral dose of MIMBP (200 mg/kg in gum acacia, for 4 weeks) and subjected to chronic swimming exercise.

Group VI: MIMBP (300 mg/kg) + exercise: Rats were administered a single daily oral dose of MIMBP (300 mg/kg in gum acacia, for 4 weeks) and subjected to chronic swimming exercise.

Group VII: PMIMBP (100 mg/kg) + exercise: Rats were administered a single daily oral dose of PMIMBP (100 mg/kg, for 4 weeks) and subjected to chronic swimming exercise.

Group VIII: PMIMBP (200 mg/kg) + exercise: Rats were administered a single daily oral dose of PMIMBP (200 mg/kg for 4 weeks) and subjected to chronic swimming exercise.

Group IX: PMIMBP (300 mg/kg) + exercise: Rats were administered a single daily oral dose of PMIMBP (300 mg/kg, for 4 weeks) and subjected to chronic swimming exercise.

The animals were subjected to chronic swimming exercise as per the reported protocol (Matsakas et al., 2006) with minor modifications. All groups (except Groups I and II) were subjected to intensive daily swimming exercise for 4 weeks, 5 days per week with gradually increasing intensity and duration of swimming stimulus. The rats swam together for 60–90 minutes per day during the 1st week in a transparent, cylindrical polypropylene tank (40 × 24 cm^2) containing water to a level of 30 cm at 25 ± 2°C. Group swimming induces vigorous muscle activity as the rats climb over each other unlike when swimming alone. An external weight corresponding to 1.5% of the animal's body weight was attached to the base of the tail during the 2nd week and the rats swam for 90 minutes per day. Subsequently during the 3rd week and 4th week, the rats swam for 90 minutes per day with a tail-weight that increased weekly to 3% and 6% of the animal's body weight, respectively. After the initial vigorous movements during the first few minutes of water exposure, the rats balance themselves on their

tails by rotating their bellies thereby reducing the swimming activity. Therefore in order to provide continuous stimuli during chronic swimming, the attached external weight was gradually increased each week. The MIMBP and PMIMBP were administered daily each morning before the swimming exercise.

4B.1.6.1.3. Tissue sampling

Upon completion of the 4th week of swimming exercise, the rats were immediately sacrificed by cervical dislocation. The gastrocnemius muscles were excised from hind limbs of the rats, frozen quickly in liquid nitrogen and stored at −80 ˚C. The isolated muscle tissue samples were weighed and divided into three parts. In total between 30 mg and 40 mg of tissue was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) studies, another portion of tissue (500 mg) was used for analysis of oxidative markers and (1000 mg) mitochondrial enzyme determination. The gastrocnemius muscles were allocated for histopathological analysis.

4B.1.6.1.4. Biochemical analysis

The isolated gastrocnemius muscle tissues were cut into small pieces, placed in a chilled sucrose solution (0.25 M) and homogenized in Tris buffer (pH 7.4, 10 mM) to a concentration of 10% w/v in a tissue homogenizer (Remi Motors, Mumbai, India). In order to release the soluble proteins, prolonged homogenization under hypotonic condition was designed to disrupt the structure of cells. The homogenates were subjected to centrifugation at 13,000 xg at 4˚C for 25 minutes using an Eppendorf 5810-Rhigh speed cooling centrifuge. The clear supernatant was subjected to analysis of lipid peroxidation, Superoxide dismutase (SOD), Glutathione (GSH) reduction, total protein content and nitrite level (NO) as mentioned below:

4B.1.6.1.4.1. Determination of SOD contents:

SOD assay was performed according to reported method (Misra and Fridovich, 1972). Briefly, the tissue homogenate (0.5 ml) was diluted with distilled water (0.5 ml) to which an ice cold ethanol (0.25 ml) and ice-cold chloroform (0.15 ml) were added. The mixture was subjected to cyclo-mixer and centrifuged at 2500 rpm at 4°C for 15 min.

To the supernatant (0.5 ml), carbonate buffer (1.5 ml) and EDTA solution (0.5 ml) were added. The reaction was initiated by the addition of epinephrine (0.4 ml) and the change in optical density / min was measured at 480 nm against reagent blank. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. SOD activity is expressed as units / mg protein.

4B.1.6.1.4.2.Determination of GSH contents:

The GSH assay was performed according to reported method (Moron et al., 1979). Briefly, equal volumes of tissue homogenate (supernatant, 2.0 ml) and the trichloroacetic acid (TCA, 20% w/v, 2.0 ml) were mixed. The mixture with precipitated fraction was centrifuged at 2500 rpm at 4°C for 15 min. To the supernatant (0.25 ml), DTNB reagent (2.0 ml) was added. The final volume was made up to 3.0 ml using phosphate buffer. The colored mixture was measured at 412 nm against reagent blank. The amount of reduced glutathione is expressed as μg of GSH / mg protein.

4B.1.6.1.4.3. Determination of malondialdehyde (MDA) content:

MDA levels in the tissue were determined according to reported method (Slater and Sawyer, 1971). Briefly, the tissue homogenate (supernatant, 2.0 ml) was mixed with the freshly prepared TCA (10% w/v, 2.0 ml). The mixture was allowed to stand in an ice bath for 15 min and the precipitate formed was separated by centrifugation at 2500 rpm at 4°C for 15 min. The clear supernatant solution (2.0 ml) was mixed with freshly prepared thiobarbituric acid (TBA, 2.0 ml). The resulting solution was heated in boiling water bath for 10 min followed by immediate cooling in an ice bath for 5 min. The colored mixture was measured at 532 nm against reagent blank. The results are expressed as nM of MDA / mg protein.

4B.1.6.1.4.4.Determination of nitrite (NO) level:

NO level from the tissue was estimated in terms of nitrite using the acidic Griess reaction based on reduction of nitrate to nitrite by vanadium trichloride, according to reported method (Miranda et al., 2001). Briefly, to the tissue homogenate supernatant

(0.5 ml), vanadium trichloride (0.5ml) was added. To the mixture Griess reagent [N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1% w/v) and sulfanilamide (2% w/v) in hydrochloric acid, (5% v/v)] was added. The pink colored solution comprising azo-product was measured at 543 nm and the results were expressed in µg/ml. A calibration curve using sodium nitrate standard solution was performed.

4B.1.6.1.4.5. Determination of protein content:

Protein concentration was estimated according to the reported method (Lowry et al., 1951). Briefly, the tissue fraction aliquot (0.1 ml) was mixed with sodium hydroxide solution (0.1 M, 0.8 ml) and Lowry C reagent (5 ml) in test tube. The solution was allowed to stand for 15 min. To the mixture Folin phenol reagent (0.5 ml) was added and subjected to mixing on vortex. Color developed was measured at 660 nm against reagent blank containing distilled water instead of sample. Bovine serum albumin (BSA) was used as standard and different concentrations (40-200 µg) were processed similarly to develop calibration curve. The results were expressed as mg of protein / g of wet tissue.

4B.1.6.1.5. Mitochondrial enzymes estimation

4B.1.6.1.5.1. Mitochondrial isolation:

Gastrocnemius muscle mitochondrial isolation was performed according to a previously described methods (Berman and Hastings, 1999) and (Rosenthal et al., 1987). Briefly, the gastrocnemius muscle (1000 mg) was homogenized in buffer and the homogenates were centrifuged at 13,000xg at 4˚C for5 minutes. Pellets were suspended in isolation buffer with ethylene glycol tetra acetic acid (EGTA) and spun at 13,000 gat 4˚C for 5 minutes. The resulting supernatants were transferred into new tubes and topped off with isolation buffer with EGTA and respun at 13,000 g at 4˚C for 10 minutes. Pellets containing pure mitochondria were re-suspended in isolation buffer without EGTA and subjected to mitochondrial enzyme activity measurement. Mitochondrial enzymes complex-I (NADH dehydrogenase), complex-II (succinate dehydrogenase) and complex IV (cytochrome oxidase activities) were measured spectrophotometrically as described below.

4B.1.6.1.5.2. Complex-I (NADH dehydrogenase activity)

Complex-I was measured spectrophotometrically according reported method (King and Howard, 1962). The method involves catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome C. Glycyl glycine (0.2 M) was prepared by dissolving 335 mg in phosphate buffer saline (10 ml) with the pH equal to 8.5.NADH (6 mM) was prepared by dissolving 42.6 mg in glycyl glycine buffer (10 ml). Cytochrome C (1.05 mM) was prepared by dissolving 13.65 mg in double distilled water (1ml). Sodium bicarbonate (0.02 M) was prepared by dissolving 16.8 mg in double distilled water (10 ml). The reaction mixture contained glycyl glycine buffer (0.2 M, pH 8.5, 350 µL), NADH (6 mM, 100 µL) in glycyl glycine buffer (2 mM), cytochrome C (10.5 mM, 100 µL) and double distilled water (2.4 ml) and sodium bicarbonate (20 µL). The reaction was initiated by addition of solubilized mitochondrial sample (10µL) and followed absorbance change at 550 nm for 180 seconds. Mitochondrial Complex I activity was calculated as follows:

Mitochondrial Complex I (i.e. n mole of NADH oxidized /min/mg Protein

= [Change in optical density per minute x 0.262 x 3 x 10^3] / Amount of protein (mg) in 10µL

4B.1.6.1.5.3. Complex-II (succinate dehydrogenase (SDH) activity)

SDH was measured spectrophotometrically according to reported method (King, 1967). The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. Sodium dihydrogen phosphate (3.12 gm) was dissolved in double distilled water (100 ml). Separately disodium hydrogen phosphate (2.83 gm) was dissolved in double distilled water (100 ml). The solutions were mixed and pH was adjusted to 7.8. Succinic acid (0.2M) was prepared by dissolving 700 mg in double distilled water (10 ml). Potassium ferricyanide (0.03M) was prepared by dissolving 19.6ml in double distilled water (2ml). Bovine serum albumin (BSA, 1%) was prepared. The reaction mixture contained phosphate buffer of pH 7.8 (0.2 M, 1.5 ml), BSA (1%, 300 µl), succinic acid (0.6 M,200 µl) and potassium ferricyanide (0.03 M, 25 µl) in double distilled water (1.75 ml). The reaction was initiated by addition of

mitochondrial sample and absorbance change was followed at 420 nm for 180 seconds. The mitochondrial complex II activity was calculated as follows:

Mitochondrial Complex II (mM of succinate dehydrogenase /mg protein) =

[Change in optical density per minute x 3.8 x 0.435 x 10^6] / Amount of protein (mg) in 25µL x 1000

4B.1.6.1.5.4. Complex-III (MTT assay)

The MTT assay was based on the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess the activity of mitochondrial respiratory chain in isolated mitochondria as determined by reported method (Liu et al., 1997). Solution of MTT was prepared by dissolving 10 mg in phosphate buffer saline (10 ml). Briefly, the mitochondrial sample (100 µl) was incubated with MTT (10 µl) for 3 hours at 37 ^{0}C on an ELISA plate in a humidified atmosphere of 5% CO_2 + 95% air at 37°C for 3 h. The blue formazan crystals were solubilized with dimethylsulfoxide (200 µl) and measured by an ELISA reader at 580 nm filter. The value of the mitochondrial complex III (i.e. number of viable cells /well) was calculated for each sample using the standard curve by extrapolation of the optical density values.

4B.1.6.1.5.5. Complex IV (cytochrome oxidase assay)

Cytochrome oxidase activity was performed according to reported method (Sottocasa et al., 1967). Sodium phosphate buffer (0.05M) was prepared by dissolving sodium dihydrogen phosphate (1.17 gm) in double distilled water (100 ml). Disodium hydrogen phosphate (1.06 gm) was dissolved in double distilled water (100 ml). Both the solutions were mixed and pH adjusted to 7.4.Cytochrome C (0.03 mM) was prepared by dissolving 39 mg in phosphate buffer (10 ml). Hydrochloric acid (100 mM) was prepared by dissolving concentrated hydrochloric acid (0.43 ml) in double distilled water (50 ml). Cytochrome C (100 ml) was reduced by addition of sodium borate crystals (10 mg) and neutralized with the hydrochloric acid (100 mM) till the pH was 7. Further the reduced Cytochrome C (100 µl) was added to phosphate buffer

(700 µL). To this the mitochondrial sample (10 µl) was added and change in optical density was measured at 550 nanometer for 180 seconds. The mitochondrial complex IV activity was calculated as:

Mitochondrial Complex IV (mMole of cytochrome-C oxidized /min/mg Protein) = [Change in optical density per minute x 3 x 10^8]/ 60 x 29.5 x Amount of protein (mg) in 10µl]

4B.1.6.1.6. RNA extraction and RT-PCR analysis

The RNA extraction and RT-PCR analysis was performed using a standard protocol provided by Biotools BandM Labs. Briefly, the isolated gastrocnemius muscle tissue (30–40 mg) was disrupted in liquid nitrogen using a mortar and pestle. To this, lysis buffer (350 µL) and mercaptoethanol (3.5 µL) were added followed by vigorous vortexing. The lysate was loaded into a filtering column in a collection tube and centrifuged for 1 minute at 11,000xg. The clarified lysate was mixed with 70% ethanol (350 µL) and loaded into the RNA binding column in a collection tube followed by centrifugation for 30 seconds at 11,000xg. Desalting buffer DBR (350 µL) and rDNase reaction mixture (95 µL) were added and incubated at room temperature for 15 minutes. Following successive washings with wash buffer, RNA was resuspended in ribonuclease-free water (60 µL) and centrifuged. The elute containing pure RNA was stored at−80◦C until analysis.

4B.1.6.1.6.1. cDNA preparation

Single-stranded cDNA was synthesized from total cellular RNA using RT-PCR. Briefly, total RNA (2 µL) was treated with100 mM magnesium sulfate solution (10 µL), primers (5 µL), and PCR astringent (12 µL). The volume was made up to 50 µL with nuclease free water. The primers were myostatin (5-ATCTGAGAGCCGTCAAGACTCC-3,5-CAGTCAAGCCCAAAGT-CTCTCC-3 base pair-340) and β-actin (5-GGCATCGTGATGG-ACTCCG-3, 5GCTGGAAGGTGGACAGCGA-3base pair-760) both synthesized by Amnion Biosciences Pvt. Ltd. The amplification of β-actin served as a control for sample loading and integrity. PCR products were detected by electrophoresis on a 1.5%

agarose gel containing ethidium bromide. The size of amplicons was confirmed by using a 100 bp ladder (Amnion Biosciences Pvt. Ltd.) as a standard size marker. The amplicons were visualized and images were capture during a gel documentation system (Alpha Innotech Inc., USA). Gene expression was assessed semiquantitatively by generating densitometry data for band intensities in different sets of experiments and analyzing the gel images using the software Image J program (version 1.33, USA). The band intensities were compared with constitutively expressed β-actin. The intensity of myostatin mRNAs was standardized against that of the β-actin mRNA from each sample and results were expressed as the myostatin mRNA/β-actin mRNA ratio.

4B.1.6.1.7. Histopathological analysis

The excised gastrocnemius muscle tissues were stored in 10%formalin for 24 hours. The specimens were dehydrated and placed in xylene for 1 hour (3 times) and later in ethyl alcohol (70%, 90%, and 100% respectively) for 2 hours. Tissue specimens were cut into sections of 3–5 m thickness and were stained with hematoxylin and eosin (HandE). The specimens were mounted on a slide using a distrene phthalate xylene (DPX) medium. The sections were examined under a light microscope.

Statistical analysis:

The pollen samples screened for nutritional and chemical evaluations were analyzed in triplicate unless otherwise stated and the results were expressed as mean ± SD. Data for *in vitro* antioxidant activity were analyzed by two way ANOVA followed by Bonferroni post-test. $P<0.05$ was considered significant. Data values for preclinical investigations were expressed as the mean ± standard error mean (SEM). All the data analysis was performed using software (version 5.0, Graph Pad, San Diego, CA, USA). Data of biochemical parameters were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for post hoc analysis. A p value < 0.05 was considered to be statistically significant. Gel images were analyzed using Image J 1.37, NIH, USA software.

4B.2. Results and Discussion:

Preliminary study:

In order to ascertain the effect of mechanical grinding on pollen, the MIMBP was subjected to ball milling process. Upon milling, there was observed adherence of sticky pollen mass to the walls of vessel and to the beads. Further the darkening of the milled pollen mass was observed which might be attributed to melting or degradation of lipid components of pollenkit. The milled pollen when extracted with ethanol (99%; 20 mL) at 70± 0.5℃ for 30 min, demonstrated total polyphenols content of 1751.32 ± 66.84 mg/ 100g which was comparable with that of neat MIMBP. Figure 4.7 denotes SEM image for the milled MIMBP sample. The image demonstrates intactness of the pollen shell indicating its resistant nature against ball milling process.

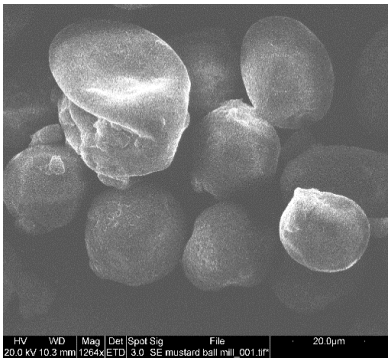


Figure 4.7. SEM image of milled MIMBP

Processing of pollen with an edible lipid-surfactant mixture:

All the processed pollen samples were yellowish brown in color and got readily dispersed in distilled water upon vortexing before analysis. Table 4.3 depicts total polyphenols content for the MIMBP samples processed with different lipid-surfactant mixtures. Further the table 4.4 shows total polyphenols content for processed pollen using different weight ratios of Captex 355 and Tween 80.

Table 4.3. Total polyphenols content for MIMBP processed with different lipid-surfactant systems

No.	Lipids (with Tween 80)	Total Polyphenol content (mg/100g)
1	Captex 355	2134.76 ± 127.29
2	Lauroglycol FCC	1665.35 ± 95.29**
3	Labrafil M 1944	1431.90 ± 73.25***

[MIMBP: Lipid: Tween 80 at weight (mg) ratio of 1000:500:500 (Mean ± SD; n=3)]

Table 4.4. Optimization of Captex 355: Tween 80 ratio

No.	Captex 355:Tween 80 (mg:mg)	Total Polyphenol content (mg/100g)
1	500:500	2134.76 ± 127.29
2	500:750	3123.35 ±163.44
3	750:500	2534.34 ± 138.61
4	750:750	2758.99 ±145.62

[Captex 355 and Tween 80 screened at respective weight (mg) ratios per 1000 mg of MIMBP (Mean ± SD; n=3)]

Lipid based systems in the form of mixture of oil and surfactants have been reported to improve the solubility and bioavailability of poorly soluble actives (Pouton, 2000). Several lipid excipients comprising medium chain triglycerides (MCTs) have been documented for improving the poor solubility of phytoconstituents and thereby increasing their extraction efficiency (Aher et al., 2009, Sathiyanarayanan et al., 2010, Ketkar et al., 2011). Also, surfactants, on account of their amphiphilic nature comprising both hydrophobic and hydrophilic moieties, have been employed for extraction of several phytocompounds especially phenolics (Gilda et al., 2010, Yazdi, 2011, Hosseinzadeh et al., 2013). Tween 80, a nonionic, less toxic and stable surfactant comprising polyoxyethylene-(20)-sorbitan monooleate, has proven to be an efficient extractant for polyphenols from apple pomace (Ajila et al., 2011).Therefore, considering the ability of MCTs and Tween 80 to improve solubility and extraction efficiency, different edible lipids (Captex 355, Labrafil M 1944, Lauroglycol 90) in combination with Tween 80 were screened for processing of neat MIMBP in view of improving the extraction of polyphenols entrapped within pollen shell.

As depicted in table 4.3, amongst different lipids-surfactant combinations screened, the Captex 355: Tween 80 system demonstrated highest yield of total polyphenols content. Therefore the system was further subjected to random optimization of lipid to surfactant ratio by varying amounts of Captex 355 and Tween 80 at constant weight of MIMBP (Table 4.4). The composition of lipid-surfactant mixture was found to be decisive in the extraction of polyphenols from pollen. Based on the highest total polyphenols content (3123.35 ±163.44 mg GAE/100g) for the pollen sample processed with Captex 355:Tween 80 at a weight ratio of 500:750, was selected for further studies (hereafter referred to as PMIMBP).

HPLC analysis showed that the PMIMBP comprised 8.89 ± 0.31 mg/100g and 13.02 ± 0.25 mg/100g of kaempferol and quercetin, respectively. Figure 4.8 displays chromatogram for blank solution prepared using Captex 355:Tween 80 mixture without pollen sample. The lipid-surfactant matrix did not show any peak interfering with that of the analytes of interest at selected wavelength thus proving it to be a simple matrix suitable for analysis. This can be attributed to the composition of Captex 355 comprising mixture of triacylglycerols which demonstrate UV absorption at lower wavelength (205-215 nm) while Tween i.e. polysorbate 80 does not contain a UV chromophore therefore shows little absorbance in the UV range (Holcapek et al., 2005). Figure 4.9 illustrates the stacked view of representative chromatograms for the standards and PMIMBP sample solution.

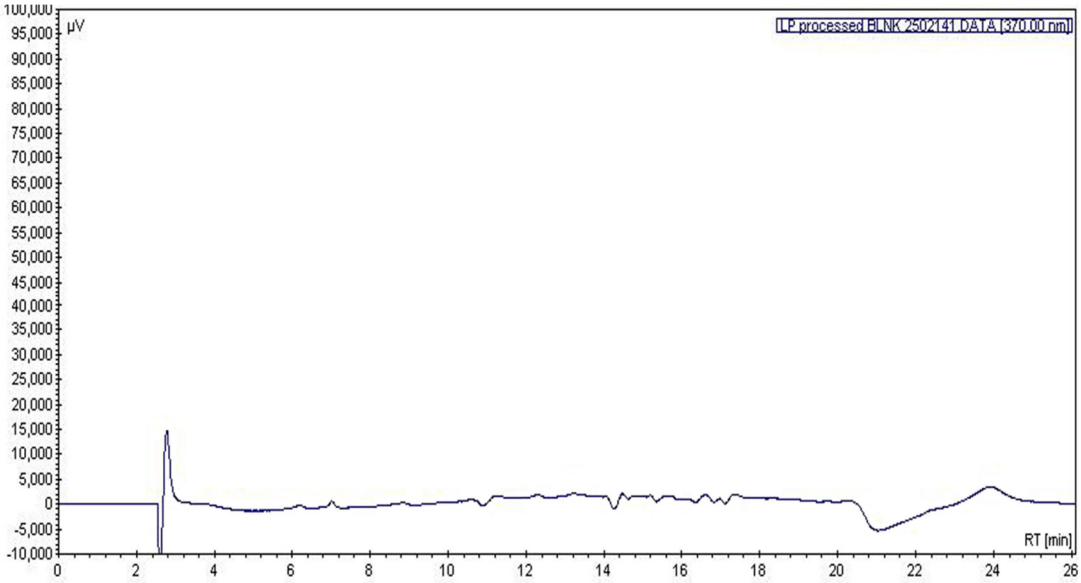


Figure 4.8. Representative chromatogram obtained for the blank lipid-surfactant solution.

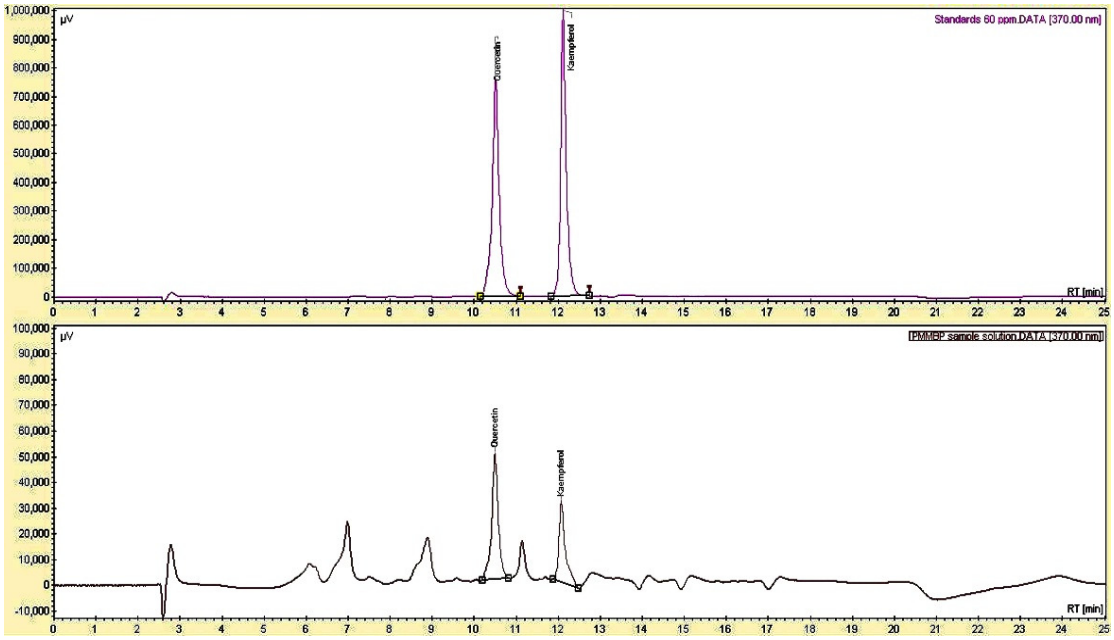


Figure 4.9. Stacked view of representative chromatograms obtained for the standards and the PMIMBP sample solution

As mentioned in table 4.1, the MIMBP when subjected to a conventional extraction method in ethanol at 70°C for 30 minutes, obtained polyphenols of about 1828.61±37.4 mg GAE/100g. Furthermore, the MIMBP was found to contain flavonoids kaempferol (6.54 ± 0.05 mg/100gm) and quercetin (5.14 ± 0.04 mg/100g). The extraction of polyphenols from herbal drug matrices is influenced by several factors such as temperature, extraction time, solvent-to-solid ratio, type of solvent, etc (Ajila et al., 2011, Dent et al., 2013). In the case of MIMBP the tough pollen shell hinders the process of extraction due to its rigid composition and insoluble nature. In the current study, observed higher yields of polyphenols and flavonoid aglycones for PMIMBP can be attributed to the properties of Captex 355 and Tween 80, respectively. Captex 355 is a MCT obtained by esterification of glycerin and fatty acids (mainly caprylic and capric acid) (Prajapati et al., 2012). It exhibits high lipophilicity and thereby possesses an ability to engulf polyphenolic compounds of wide range of polarity. It also contributes to provide stability to polyphenols against oxidation (Ajila et al., 2011).Tween 80, on account of its solubilizing effect, has contributed to the enhanced extraction of polyphenols in two possible ways: (1) by reducing the surface tension there by enhancing the release of entrapped polyphenols and (2) by facilitating

increased contact between polyphenols and the lipid mixture which ultimately solubilizes in the aqueous phase (Ajila et al., 2011). Initial stage in the process of extraction is penetration of the extractant into a herbal matrix by wetting of substances present within the cells. Surfactants reduce surface tension and facilitate wetting and swelling of the plant material which intensifies the process of mass transfer resulting in improved extraction of active substances. Similar improvements in the extraction of the flavonoid rutin from *Japanese pagoda* tree buds by using surfactants has been reported (Hosseinzadeh et al., 2013). Increased extraction of total polyphenols and flavonoid aglycones for PMIMBP can be attributed to the enhanced solubilization of polyphenolic moieties in the oil-surfactant mixture. The hydrophilic polyoxyethylene chain of Tween 80 together with the lipophilic caprylic-capric triglyceride composition of Captex 355 provides good solvent properties for engulfing the polyphenolic moieties. Similar improvements in the solubility of flavonoid quercetin in the mixture of MCT Capmul MCM and Tween 20 has been documented. (Jain et al., 2013)

Scanning electron microscopy:

Figure 4.10 (A and B) depict SEM images for MIMBP and PMIMB (respectively). The SEM analysis showed subolate to subprolate shape and medium trizonocolpate along with ambtrilobed fossaperture characteristic to MIMBP. The exine part of PMIMBP was found to be partially ruptured which may be as a result of the process of vigorous trituration in the presence of a lipid-surfactant mixture.

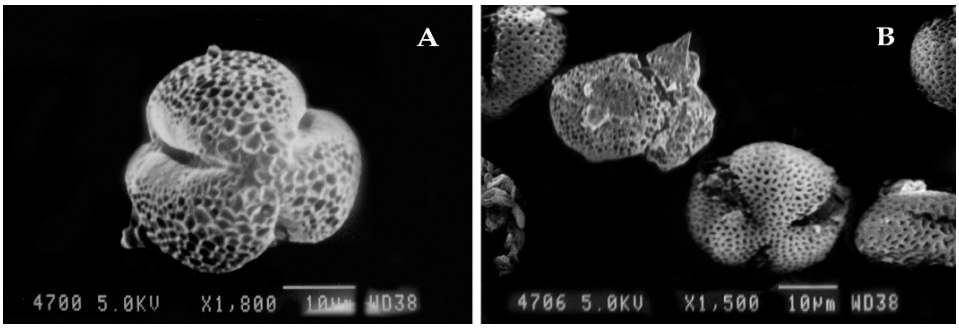


Figure 4.10. SEM image for (A) MIMBP and (B) PMIMBP samples

The trituration was found to be an important step for uniform mixing and homogenization of the lipid-surfactant mixture with the MIMBP inducing partial rupture of the pollen coat (as observed in Figure 4.10B). This might have contributed

to intensify the mass transfer of polyphenols into the lipid matrix improving the extraction efficiency. The rigid sporopollenin matrix of the pollen shell (comprising of polyterpene) is reported to be solubilized in to a mixture of a hydrolysable ester such as Tween and a hydrophilic solvent (Maack A, 2007).This suggests possible solubilization of the sporopollenin components of pollen shell into the lipid-surfactant mixture thereby contributing to enhance mass transfer of polyphenolics into the lipid matrix.

***In vitro* bio-accessibility of polyphenols from MIMBP and PMIMBP:**

Assessment of '*in vitro* bio-accessibility' provides a prediction tool for nutritional efficiency of food material (Fernandez et al., 2009). It is documented that polyphenols released from solid food matrices reflect potentially bioavailable components of the foods for exerting beneficial effects (Tagliazucchi et al., 2010, Bouayed et al., 2011). Therefore *in vitro* bio-accessibility of polyphenols from MIMBP and PMIMBP was determined. The bioavailability of dietary constituents depends mainly on their bio-accessibility and digestive stability along with their efficiency of transepithelial passage (Tagliazucchi et al., 2010). Most of the polyphenols including flavanols, hydroxybenzoic acids, hydroxycinnamic acids, stilbenoids, etc have been reported to be stable at the acidic pH of gastric environment while demonstrate degradation in alkaline intestinal environment (Tagliazucchi et al., 2010). Therefore the bio-accessibility of polyphenols from MIMBP and PMIMBP was studied in simulated gastric acid fluid (SGF). Equation for calibration curve for gallic acid in SGF was found to be as follows: $y = 0.004x + 0.002$; with $R^2 = 0.996$.

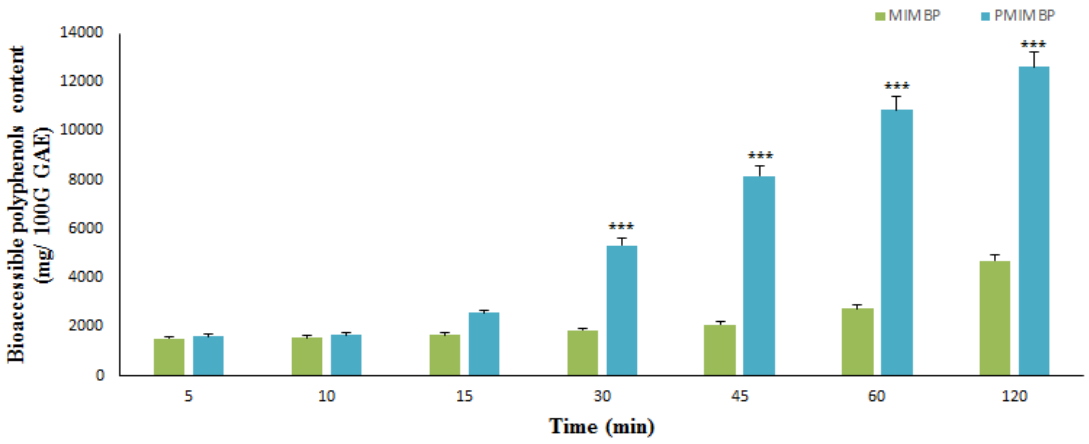


Figure 4.11. *In vitro* bio-accessibility of total polyphenols from MIMBP and PMIMBP at different time intervals

Table 4.5. Equations defining the release of polyphenols as a function of time

No	Sample	Equation obtained	R^2
1	MIMBP	$y = 1071.9e^{0.1707x}$	0.825
2	PMIMBP	$y = 931.67e^{0.3967x}$	0.958

Figure 4.11 depicts bio-accessible amount of total polyphenols released from the food matrices (MIMBP and PMIMBP) in SGF at different time intervals. As disclosed in table 4.5, the regression analysis showed exponential release kinetics for both the pollen samples over a period of 2 hrs. The MIMBP released polyphenols with a factor of 0.1707 while the PMIMBP showed about 2.33 fold increase in the rate of polyphenols release with a factor of 0.3967.

The slow release of polyphenols through MIMBP can be attributed to the insoluble nature of pollen coat. It is documented that the release of active constituents entrapped within pollen shells occurs by passive diffusion (Diego-Taboada et al., 2014). Therefore the release of polyphenols from MIMBP depends on penetration of gastric fluid inside the pollen shell through germination pores and diffusion of the polyphenols into the gastric fluid. The cumulative amount of polyphenols released in SGF from MIMBP after 2 hrs was found to be 16096.9 ± 29.17 mg/100 gm.

PMIMBP demonstrated comparable yield of bio-accessible polyphenols for initial 15 min period with that of MIMBP. This could be attributed to the controlled hydration of PMIMBP due to lipid: surfactant matrix surrounding the pollen shell. This induces

gelation within the confined space of dialysis bag, retarding the release of polyphenols into the gastric fluid in the initial phase. Consequently the solubilized polyphenols diffuse through the gel phase into the gastric fluid thereby demonstrating significant ($p < 0.001$) improvement in the yield of bio-accessible polyphenols at all the time intervals above 15 min. The cumulative amount of polyphenols released in SGF from PMIMBP after 2 hrs was found to be 42793.1 ± 84.85 mg /100 gm respectively. These findings suggests about 2.66 fold increase in the bio-accessible polyphenols content upon processing of MIMBP with edible lipid-surfactant composition.

The microscopic images were captured for MIMBP and PMIMBP before and after the *in vitro* gastric digestion exercise. The neat MIMBP when put in the simulated gastric fluid, there was apparent swelling or bulging of the pollen (Figure 4.12.A) which might be attributed to the elastic nature of pollen wall and the entry of simulated gastric fluid in to the shell. With progression of digestion process the surrounding gastric fluid entered the pollen shell through germination pores, and induced diffusion of minimal amounts of inner contents (Figure 4.12.B and C). The PMIMBP, when brought in contact with the simulated gastric fluid also demonstrated bulging of the pollen shell. Moreover there was observed altered surface texture and ornamentation of the pollen shell. (Figure 4.12.D). Upon completion of the digestion process (120 min), there was observed softening and complete disruption of the exine part with evident release of inner contents through pores. (Figure 4.12E and F). These findings confirms effective breaking of the pollen wall for PMIMBP suggesting improved digestibility of same.

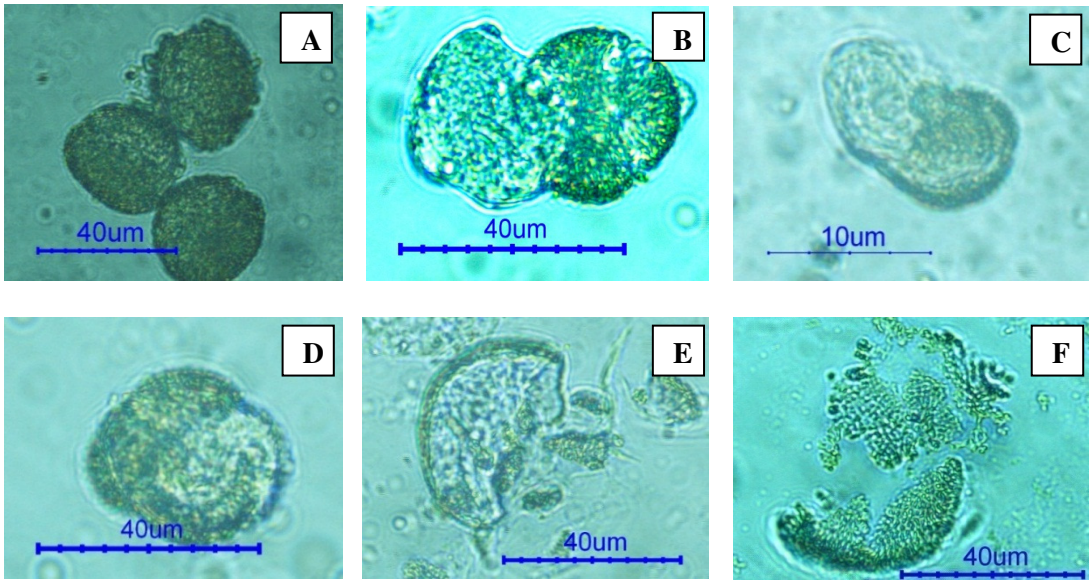


Figure 4.12. Light microscopic images for A) MIMBP in SGF at 0 min; B) and C): MIMBP in SGF at 120 min. D) PMIMBP in SGF at 0 min; E) and F) PMIMBP in SGF at 120 min

Preclinical investigation:

Effect of MIMBP and PMIMBP on exercise-induced oxidative stress muscular implications in Wistar rats

Acute oral toxicity test:

Table 4.6 shows survival data for the acute toxicity study of MIMBP and PMIMBP. No mortality was observed in any group at the administered doses. Further no signs or symptoms of toxicity were observed during the entire period. Both MIMBP and PMIMBP were found to be safe up to 2000 mg/kg.

Table 4.6. Acute oral toxicity (Swellam et al.) study for MIMBP and PMIMBP: Survival data

Treatment (dose in mg/kg)	Sex	Dead/Total	Dead (%)	Toxicity symptoms/ Adverse effects
Vehicle control	Male	0/5	0	None
	Female	0/5	0	None
MIMBP (55)	Male	0/5	0	None
	Female	0/5	0	None
MIMBP (175)	Male	0/5	0	None
	Female	0/5	0	None
MIMBP (550)	Male	0/5	0	None
	Female	0/5	0	None
MIMBP (1750)	Male	0/5	0	None
	Female	0/5	0	None
MIMBP (2000)	Male	0/5	0	None
	Female	0/5	0	None
PMIMBP (55)	Male	0/5	0	None
	Female	0/5	0	None
PMIMBP (175)	Male	0/5	0	None
	Female	0/5	0	None
PMIMBP (550)	Male	0/5	0	None
	Female	0/5	0	None
PMIMBP (1750)	Male	0/5	0	None
	Female	0/5	0	None
PMIMBP (2000)	Male	0/5	0	None
	Female	0/5	0	None

Effect on bodyweight and relative gastrocnemius muscle weight:

Table 4.7 discloses effect of MIMBP and PMIMBP on chronic swimming induced alteration in body weight and gastrocnemius muscle weight in rats.

Table 4.7. Effect of MIMBP and PMIMBP on chronic swimming induced alteration in body weight and gastrocnemius muscle weight in rats

Treatment	Body weight (g)	Absolute gastrocnemius muscle weight (g)	Relative gastrocnemius muscle weight[(a)]
Normal	252.60 ±4.22	1.82 ±0.07	0.0072 ± 0.00028
Exercised control	182.40 ±2.63###	1.19 ±0.09###	0.0064 ± 0.00048##
MIMBP (100) + Exercise	199.00 ±2.42	1.27 ±0.15	0.0064 ± 0.00076
MIMBP (200) + Exercise	204.40 ±5.62*	1.35 ±0.08	0.0066 ± 0.00038
MIMBP (300) + Exercise	217.80 ±2.87***	1.57 ±0.04*	0.0072 ± 0.00012*
PMIMBP (100) + Exercise	213.80 ±1.93***	1.31 ±0.12	0.0061 ± 0.00058
PMIMBP (200) + Exercise	219.60 ±3.20***	1.54 ±0.06*	0.0070 ± 0.00023
PMIMBP (300) + Exercise	226.40 ±9.01***	1.70 ±0.07***	0.0076 ± 0.00062**
Per se	248.40 ± 7.38	1.84 ±0.09	0.0076 ± 0.00056

Data are expressed as mean ± S.E.M. Data was analyzed by one-way ANOVA followed by Dunnett's test ## P <0.01, ### P <0.001 as compared to normal rats. * P <0.05 and *** P <0.001 as compared to exercise control rats. [(a)]Relative gastrocnemius muscle weight represents the ratio of absolute gastrocnemius muscle weight to the body weight.

There was observed a significant decrease ($p < 0.001$) in the body weight of exercised control rats as compared to that of normal rats. A significant reduction ($p < 0.01$) in the relative gastrocnemius muscle weight of exercised control rats was observed in comparison to the normal group. Rats administered with MIMBP (200 mg/kg and 300 mg/kg) showed significant ($p < 0.05$ and $p < 0.001$) and dose dependent inhibition of decrease in body weight when compared to exercised control rats. Relative weight of the gastrocnemius muscle increased significantly ($p < 0.05$) in MIMBP (300 mg/kg) treated rats when compared to that of exercised control rats. When compared with exercised control rats, PMIMBP (100 mg/kg, 200 mg/kg, and 300 mg/kg) treated rats showed a significant increase ($p < 0.001$) in bodyweight whereas the relative gastrocnemius muscle weight was significantly increased ($p < 0.05$) in the PMIMBP

(300 mg/kg) treated rats. Per se treated animals did not show any significant change in body weight and relative gastrocnemius muscle weight when compared to that of normal rats.

Effect on antioxidant markers:

Table 4.8 records alterations in SOD, GSH, MDA, NO and total protein levels in gastrocnemius muscle of the rats from different groups

Table 4.8. Effect of MIMBP and PMIMBP on chronic swimming induced alteration in SOD, GSH, MDA, NO and total protein in gastrocnemius muscle in rats

Treatment	SOD (U/ mg of protein)	GSH (µg/ mg of protein)	MDA (nm/ mg of protein)	NO (µg/ml)	Total protein (mg/gm)
Normal	23.53 ± 0.45	1.86 ± 0.14	2.98 ± 0.32	97.66 ± 8.61	18.50 ± 2.69
Exercised control	4.17 ± 0.57$^{###}$	0.22 ± 0.03$^{###}$	20.10 ± 0.65$^{###}$	512.2 ± 14.99$^{###}$	74.50 ± 7.84$^{###}$
MIMBP (100) + Exercise	6.31 ± 0.55	0.45 ± 0.06	19.54 ± 0.53	499.5 ± 11.01	63.50 ± 5.62
MIMBP (200) + Exercise	8.34 ± 0.91*	0.57 ± 0.06*	18.80 ± 0.43	464.7 ± 7.70	63.50 ± 6.96
MIMBP (300) + Exercise	10.15 ± 0.84**	0.62 ± 0.07*	17.04 ± 0.74**	444.6 ± 8.83**	47.50 ± 3.76**
PMIMBP (100) + Exercise	8.58 ± 0.71*	0.44 ± 0.03	16.84 ± 0.46**	452.5 ± 14.43**	67.5 ± 3.26
PMIMBP (200) + Exercise	14.89 ± 2.07***	0.73 ± 0.04**	13.12 ± 0.71***	383.8 ± 13.46***	43.0 ± 6.68***
PMIMBP (300) + Exercise	17.24 ± 0.69***	1.27 ± 0.13***	9.58 ± 1.09***	241.0 ± 20.00***	31.0 ± 3.58***
Per se	21.73 ± 1.49	1.69 ± 0.11	3.82 ± 0.32	93.88 ± 6.64	27.00 ± 2.15

Data are expressed as mean ± S.E.M. Data was analyzed by one-way ANOVA followed by Dunnett's test ###P < 0.001 as compared to normal rats. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared to exercise control rats

Effect of MIMBP and PMIMBP treatment on SOD and GSH levels

Significant reduction ($p < 0.001$) in SOD and GSH levels of the gastrocnemius muscle was observed in the exercised control group as compared to that of normal rats.

MIMBP (200 mg/kg and 300 mg/kg) treatment significantly inhibited SOD reduction ($p < 0.05$ and $p< 0.001$ respectively). Moreover it significantly prevented reduction in GSH levels as compared to that of exercised control rats ($p < 0.05$). PMIMBP (100 mg/kg, 200 mg/kg, and 300 mg/kg) treated rats showed a significant and dose dependent increase in SOD level as compared to that of exercised control rats ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively). PMIMBP (200 mg/kg and 300 mg/kg) treatment showed a significant and dose dependent increase in GSH levels as compared to exercised control rats ($p < 0.01$ and 0.01 and $p < 0.001$). There was no significant change in SOD and GSH levels in per se treated animals when compared with normal rats.

Effect of MIMBP and PMIMBP treatment on MDA, NO, and total protein level:
There was observed a significant increase ($p < 0.001$) in MDA, nitric oxide (NO), and total protein level in the gastrocnemius muscle of exercised control group rats as compared to normal rats. The MIMBP (300 mg/kg) treatment showed significant reduction in MDA, NO, and total protein level as compared to that of exercised control rats ($p < 0.01$). The PMIMBP (100 mg/kg, 200 mg/kg and 300 mg/kg) treatment showed a significant and dose dependent decrease in MDA and NO level as compared to vehicle rats ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). When compared with exercised control rats, treatment with PMIMBP (200 mg/kg and 300 mg/kg) showed a significant reduction ($p < 0.001$) in the total protein level. Per se treated animals did not show any significant alteration in MDA, NO, and total protein level as compared to normal rats (Table 5.8).

The observed results of biochemical analysis in terms of SOD, GSH, MDA, NO, and total protein levels confirmed an antioxidant effect of both MIMBP and PMIMBP in oxidative stress induced situations in the gastrocnemius muscle of Wistar rats. Forced swimming stress has been documented to be a continuous stressor with both psychological and physiological components (Haleagrahara et al., 2009).The exhaustive swimming stress for 4 weeks decreased the body mass and reduced relative weight of the gastrocnemius muscle in exercised control rats, which can be attributed

to a decreased fat component (Matsakas et al., 2006). During exercise, energy demand exceeds around 35 times than required at rest (Astrand and Rodahl, 1977).Therefore the oxygen intake increases greatly during muscular activity. This leads to the generation of ROS that is considered responsible for muscle fatigue during exercise. Different mechanisms which contribute to the generation of free radicals may include: (1) the elevation of intramuscular calcium during high intensity exercise which activates protease enzymes which converts xanthine dehydrogenase into xanthine oxidase. This consumes molecular oxygen instead of NAD+, and thereby produces superoxide radicals; (2) intermittent hypoxia and reoxygenation in exercised muscles during cyclic contractions and relaxations may convert molecular oxygen into a superoxide radical; (3) strenuous physical exercise may lead to temporary ischemia in the muscles exerting damage. This induces activation of leukocytes which may stimulate the production of free radicals; (4) ischemic or hypoxic conditions stimulate NO synthase activity, leading to the generation of NO radicals; and (5) the activation of phospholipase A2 releases arachidonic acid from phospholipids. Cyclooxygenase, when it reacts with arachidonic acid generates hydroxyl radicals (Astrand and Rodahl, 1977, Schneider and Oliveira, 2004). Further excessive exercise results in muscle glycogen depletion due to the preferential use of intramuscular triacylglycerol and circulating lipids by the skeletal muscle (Kiens and Richter, 1998). Moreover the increased breakdown of glycogen leads to intracellular accumulation of lactic acid which dissociates into lactate and H+. This proton accumulation due to acidosis contributes to exercise-induced oxidative stress (Westerblad et al., 2002).Enhanced levels of ROS in oxidative stressed conditions promote contractile dysfunction leading to skeletal muscle fatigue. Furthermore, the prolonged and intense exercise can induce oxidative damage to cellular constituents (Powers and Jackson, 2008). Therefore the skeletal muscles need a better antioxidant shield against potential damage that occurs during and after exercise. An antioxidant enzyme SOD existing in the mitochondria and cytosol, reduces the oxygen radical ($O_2^{\cdot}$) generated during exhaustive stress to H_2O and thus scavenges the free radical (Kandhare et al., 2014).The GSH shields tissues and cells against generated free radicals by converting H_2O_2 to H_2O. Observed reduced levels of SOD and GSH in the exercise control group of rats indicate the progression of

oxidative stress and disturbed balance between pro- and anti-oxidative enzymes in the cells. Both MIMBP and PMIMBP supplementation inhibited the reduction of SOD and GSH levels indicating restoration of the oxidative balance in the muscles. It was evident that the groups treated with PMIMBP exerted a greater increase in SOD and GSH levels when compared to groups treated with MIMBP. The elevated levels of MDA i.e., enhanced lipid peroxidation in the gastrocnemius muscle of exercised control group rats, indicate oxidative damage to the tissue (Cheesman, 1993, Thirumalai et al., 2011). Furthermore, the increased NO levels in the muscle tissues are suggestive of stressed conditions (Kandhare et al., 2012). MIMBP supplementation at a high dose of 300 mg/kg was found to be effective in reducing MDA and NO levels while the PMIMBP exerted progressive down regulation of the same.

Effect of MIMBP and PMIMBP treatment on mitochondrial complex enzymes:

Table 4.9 demonstrates effect of MIMBP and PMIMBP administration on mitochondrial complex enzyme activities in gastrocnemius muscle of the rats from different groups.

Table 4.9. Effect of MIMBP and PMIMBP on chronic swimming induced alteration in mitochondrial enzyme activity in gastrocnemius muscle in rats

Treatment	Complex I (nmole of NADH oxidized/min/ mg protein)	Complex II (mmole/ mg protein)	Complex III (MTT assay) (OD at 540 nm)	Complex-IV (nmol cyto-C oxidized/min/ mg protein)
Normal	22.34 ± 3.61	71.20 ± 4.25	0.39 ± 0.04	6071 ± 173.3
Exercised control	4.10 ± 0.75$^{###}$	14.85 ± 3.49$^{###}$	0.09 ± 0.01$^{###}$	1387 ± 431.3$^{###}$
MIMBP (100) + Exercise	4.91 ± 0.63	27.90 ± 2.66	0.10 ± 0.008	1277 ± 390.5
MIMBP (200) + Exercise	4.70 ± 1.05	31.77 ± 2.84**	0.16 ± 0.01	2598 ± 570.4
MIMBP (300) + Exercise	10.60 ± 0.82**	36.39 ± 5.05**	0.20 ± 0.008**	4498 ± 487.5**
PMIMBP (100) + Exercise	6.90 ± 0.68	20.93 ± 2.48	0.14 ± 0.01	1884 ± 727.6

PMIMBP (200) + Exercise	13.05 ± 1.85**	36.78 ± 3.57*	0.018 ± 0.02*	4068 ± 382.0*
PMIMBP (300) + Exercise	17.92 ± 1.36***	58.46 ± 3.76***	0.33 ± 0.03***	5578 ± 1104***
Per se	19.99 ± 1.59	73.07 ± 4.10	0.40 ± 0.02	5925 ± 806.1

Data are expressed as mean ± S.E.M. Data was analyzed by one-way ANOVA followed by Dunnett's test $^{###}P$ <0.001 as compared to normal rats. $*P$ <0.05, $**P$ <0.01 and $***P$ <0.001 as compared to exercise control rats

Exhaustive chronic swimming stress for 4 weeks significantly impaired mitochondrial complex enzyme activities in exercised control rats as compared to normal group rats ($p < 0.001$). MIMBP (300 mg/kg) treatment significantly restored mitochondrial Complex I, III, and IV enzyme activities as compared to the exercised control group ($p < 0.01$). However MIMBP (200 mg/kg and 300 mg/kg) produced a significant increase in mitochondrial Complex-II enzyme activity as compared to that of the exercised control group ($p < 0.01$). Furthermore, PMIMBP (200 mg/kg and 300 mg/kg) significantly and dose dependently inhibited the exhaustive swimming stress induced alteration in mitochondrial Complex I, II, III, and IV enzyme activities as compared to that of exercised control rats ($p < 0.05$ and $p < 0.001$). However, the per se group did not produce any significant alteration in mitochondrial Complex I, II, III, and IV enzyme activities as compared to normal rats.

Impaired mitochondrial function contributes to the development of oxidative stress by decreasing oxidative phosphorylation and adenosine triphosphate (ATP) generation along with a marked rise in free radicals (Kumar et al., 2011). During exercise the oxygen requirement of muscles increases. The oxygen consumed by the mitochondria in particular, undergoes one electron reduction generating superoxide radicals. The process of energy (ATP) production in mitochondria is catalyzed by the membrane bound protein complexes NADH dehydrogenase, succinate dehydrogenase, and ubiquinol cytochrome C oxidoreductase. NADH dehydrogenase is responsible for the transfer of electrons into the electron transport chain (ETC). It catalyzes the dehydrogenation of NADH generated through oxidation of numerous NADP-linked dehydrogenase reactions. The succinate dehydrogenase (SDH) is another enzyme responsible for the transfer of electrons into the ETC. It plays a key role in neuronal

energy metabolism. Significant reduction in Complex I and Complex II activities of the exercise control group of rats can be ascribed to an increased ROS which inhibits the catalytic function of the enzymes (Kumar et al., 2011, Hagl et al., 2013, Surapaneni et al., 2012). MIMBP (300 mg/kg) and PMIMBP (200 mg/kg and 300 mg/kg) prevented the attenuation of active NADH dehydrogenase. Furthermore, both MIMBP and PMIMBP prevented the attenuation of mitochondrial SDH activity. The improved activity of Complex I and II suggests there was an increase in the rate of transfer of electrons into the ETC (Surapaneni et al., 2012).Cytochrome C catalyzes electron transport from ubiquinone to cytochrome oxidase. Cytochrome oxidase in the presence of reduced cytochrome C and oxygen transfers a proton into the mitochondrial inner membrane. Significant reduced activity of Complex IV enzyme of the exercise control group of rats can be attributed to the oxidative stress induced by an increased ROS (Kumar et al., 2011, Hagl et al., 2013). MIMBP (300 mg/kg) and PMIMBP (200 mg/kg and 300 mg/kg) significantly reversed the reduced cytochrome oxidase activity. Results of a MTT assay indicate that there was a significant decline in Complex-III activity of the exercise control group of rats which reflects impaired mitochondrial respiration. Both MIMBP and PMIMBP were found effective in restoring the decline in mitochondrial respiration due to oxidative stress. These findings suggest that the MIMBP and PMIMBP render their antioxidant effect through up regulation of mitochondrial enzyme activity.

Effect on myostatin expression in gastrocnemius muscle:

Figure 4.13 demonstrates myostatin expression in gastrocnemius muscle of the rats from different groups. Significant up-regulation of myostatin mRNA expression in the gastrocnemius muscle of exercised control rats upon 4 weeks of exhaustive swimming stress was observed as compared to that of normal group rats ($p < 0.001$). MIMBP (300 mg/kg) treatment showed significant down-regulation in myostatin mRNA expression as compared to that of exercised control group ($p < 0.05$). The PMIMBP (200 mg/kg and 300 mg/kg) treatment showed significant inhibition in exhaustive swimming stress induced up-regulation of myostatin mRNA expression as compared to that of the exercised control group ($p < 0.001$). No significant alteration in the myostatin mRNA

expression of the per se group was observed when compared with that of normal group.

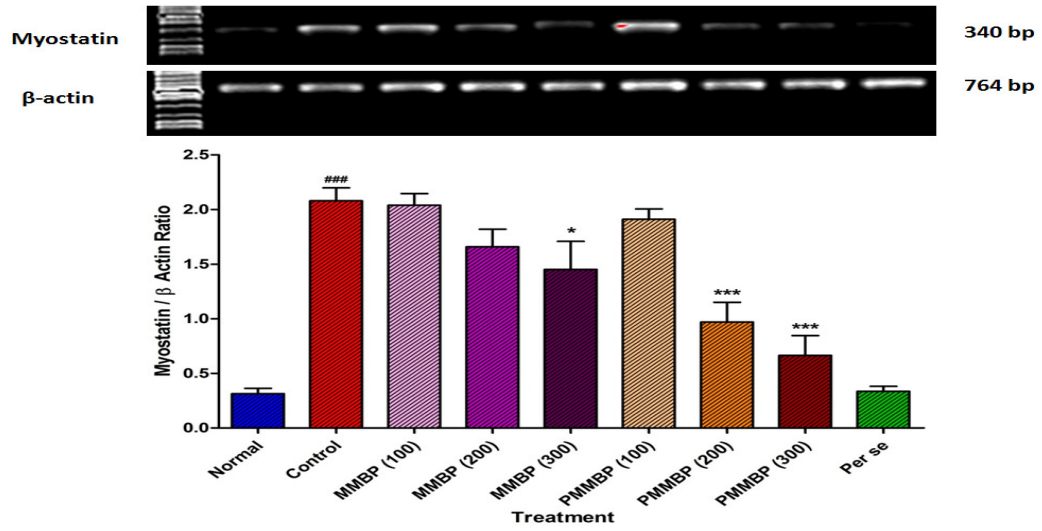


Figure 4.13. Effect of MIMBP and PMIMBP on chronic swimming induced alteration in reverse transcriptase analysis of protein expression of myostatin. Data are expressed as mean ± standard error mean. Data was analyzed by one-way analysis of variance followed by Dunnett's test.* $p < 0.05$ as compared to normal rats. †$p < 0.05$ as compared to exercise control rats

Myostatin exerts an inhibitory role on skeletal muscle development and growth (Lee, 2007, Lee, 2004). It regulates the number of myofibers formed during the development and postnatal growth of muscles. Several studies have reported alterations in the myostatin mRNA expression of rodents when exposed to rigorous exercise in the form of acute and short term swimming training (Matsakas et al., 2006), treadmill running (Wehling et al., 2000) and chronic wheel running (Matsakas et al., 2005). These evidences have shown decreased myostatinm RNA expression in the muscles of rodents when exposed to exercise or training. However in the current study a significant increase in myostatin expression at mRNA level in the exercised control group of rats when subjected to intense swimming exercise for 4 weeks was observed. This can be attributed to the development of oxidative stress in the gastrocnemius muscle due to the excessive generation of ROS during chronic swimming exercise. The elevated ROS generation activates intracellular cytokines such as tumor necrosis factor which in turn stimulate myostatin expression (Lenk et al., 2009, Sriram et al., 2011).

Similar increased myostatin mRNA and protein expression upon heavy resistance training for 12 weeks has been documented (Willoughby, 2004). Overexpression of myostatin leads to muscle wasting (Whittemore et al., 2003, Smith and Lin, 2013). It was accompanied by an observed reduction in the relative muscle and body weight of exercised control rats. This finding is in agreement with the previously stated negative role of myostatin in regulating muscle mass. Evident increases in the muscle mass and body weight of rats from Groups VI, VII, VIII, and IX as compared to that of the exercised control group confirmed the myostatin inhibitory role of both MIMBP and PMIMBP supplements suggesting their protective influence against muscle wasting conditions. Exercise induces alterations in muscle fiber morphometry and capillarization in tissues (Palstra et al., 2014). There is a need for detailed morphometrical assessment of the exercised gastrocnemius muscles to trace and quantitate the effects of MIMBP and PMIMBP supplementation. Moreover, apart from myostatin, the influence of other genes such as fibroblast growth factors(fgf-18 and fgf-20) and atrogenes involved in growth, development, and degradation of skeletal muscles (Palstra et al., 2014) need to be analyzed.

Histopathological analysis

Figure 4.14 demonstrates histopathological observations for the gastrocnemius muscles of rats from different groups.

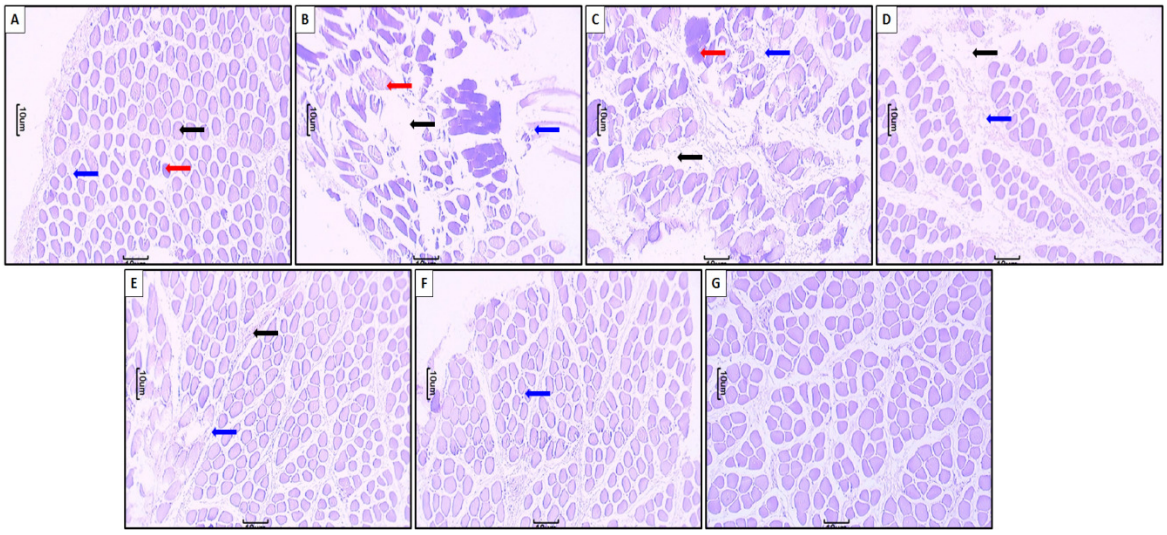


Figure 4.14. Effect of MIMBP and PMIMBP on chronic swimming induced alteration in histopathological analysis of gastrocnemius muscles. Photomicrographs of sections of gastrocnemius muscles from rats stained with hematoxylin andeosin. Gastrocnemius

muscles microscopic image of (A) normal rat, (B) exercise control rat, (C) MIMBP (200 mg/kg) + exercise rat, (D) MIMBP (300 mg/kg) + exercise rat, (E) PMIMBP (200 mg/kg) + exercise rat, (F) PMIMBP (300 mg/kg) + exercise rat, and(G) Per se treated rats (microscopic examination under 100 × light microscopy).

Histological findings of the gastrocnemius muscle of the normal group of rats stained with HandE revealed presence of bundles of muscle fibers (black arrow) separated by connective tissue perimysium (blue arrow). The connective tissue endomysium containing blood vessels (red arrow) was detected among the muscle fibers (Figure 4.14A). Gastrocnemius muscle from exercised control rats showed splitting of the myofibers (red arrow) in addition to focal areas with cellular infiltration (blue arrow) and containing myofibers characterized by fragmentation of the sarcoplasm and necrosis (black arrow). The exercised control group showed few blood vessels as compared to the normal group (Figure 4.14B). Rats treated with MIMBP (200 mg/kg) showed the presence of cellular infiltration (blue arrow), splitting of the myofibers (red arrow) and necrosis (black arrow) (Figure 4.14C). These features were halted by MIMBP (300 mg/kg) treatment (Figure 4.14D). However, PMIMBP (200 mg/kg) treated rats showed the presence of cellular infiltration (blue arrow) and necrosis (black arrow) but the structure of myofibers remained intact (Figure 4.14E). PMIMBP (300 mg/kg) treated rats showed minor infiltration of inflammatory cells (blue arrow) with a normal myofibers structure evident as compared to that of the exercised control group (Figure 4.14F).

Figure 4.14G depicts the normal architecture of a gastrocnemius muscle from per se treated animals. Qualitatively the histological investigation suggests that supplementation of PMIMBP inhibits the increase in thickness of muscle fibers (Figure 4.14 E and F) as compared to that of the exercised control group.

In summary, the findings of this study highlight the antioxidant influence of MIMBP (and PMIMBP) at a biochemical and mitochondrial level in the gastrocnemius muscle of Wistar rats. Additionally, the observed myostatin inhibitory effects suggests muscle protectant ability. Along with the polyphenols, MIMBP also comprises other nutrients such as proteins, carbohydrates, etc. These nutrients might also have contributed to the observed beneficial effects. Based on the evident improvement in

therapeutic efficiency of PMIMBP over MIMBP, it is believed that MCT and the surfactant composition of a lipid matrix might have contributed to improving the bioavailability of pollen nutrients; however systematic studies to determine their levels circulating in the blood and available at the site of absorption need to be undertaken.

4B3. Conclusion:

In conclusion, this study explores muscle protectant role of neat and processed bee pollen supplementation on exercise-induced oxidative stress implications in gastrocnemius muscle of Wistar rats. Processing of MIMBP using an edible lipid-surfactant mixture proved useful for improving the availability of engulfed nutrients to exert beneficial effects. The mitochondrial up-regulating effects of MIMBP and PMIMBP were perceived. Further myostatin inhibitory effects of MIMBP and PMIMBP were established suggesting their role in preventing muscle wasting conditions.

References:

Aher S, Biradar S, Gopu C and Paradkar A (2009). Novel pepper extract for enhanced P-glycoprotein inhibition. *Journal of Pharmacy and Pharmacology,* 61**:**1179-86.

Ajila C, Brar S, Verma M, Tyagi R and Valero J (2011). Solid-state fermentation of apple pomace using Phanerocheate chrysosporium–Liberation and extraction of phenolic antioxidants. *Food Chemistry,* 126**:**1071-80.

Almaraz AN, Campos MG, Avila RJA, Naranjo JN, Herrera CJ and Gonzalez VLS (2004). Variability of antioxidant activity among honeybee-collected pollen of different botanical origin. *Interciencia Caracas,* 29**:**574-78.

Astrand PO and Rodahl K 1977. Textbook of work physiology: physiological bases of exercise. McGraw-Hill (New York).

Berman SB and Hastings TG (1999). Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria. *Journal of neurochemistry,* 73**:**1127-37.

Bernsdorff C, Wolf A, Winter R and Gratton E (1997). Effect of hydrostatic pressure on water penetration and rotational dynamics in phospholipid-cholesterol bilayers. *Biophysical journal,* 72**:**1264-77.

Bonvehi SJ, Torrento SM and Lorente CE (2001). Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. Journal of agricultural and food chemistry, 49:1848-53.

Bouayed J, Hoffmann L and Bohn T (2011). Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. *Food Chemistry,* 128**:**14-21.

Campos M, Markham K and Proença Da Cunha A (1997). Quality assessment of bee-pollens using flavonoid/phenolic profiles. *Polyphenol Communications,* 96**:**53-54.

Campos MG, Bogdanov S, De Almeida MLB, Szczesna T, Mancebo Y, Frigerio C and Ferreira F (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research,* 47**:**154-61.

Campos MG, Webby RF, Markham KR, Mitchell KA and Da Cunha AP (2003). Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *Journal of agricultural and food chemistry,* 51**:**742-45.

Carpes ST, Begnini R, Alencar SMD and Masson ML (2007). Study of preparations of bee pollen extracts, antioxidant and antibacterial activity. *Ciência e Agrotecnologia,* 31**:**1818-25.

Cartea ME, Francisco M, Soengas P and Velasco P (2010). Phenolic compounds in Brassica vegetables. *Molecules,* 16**:**251-80.

Cheesman K (1993). Lipid peroxidation in biological systems. *DNA and free radicals. Ellis Horwood, London,* 16**:**12-7.

Dent M, Dragovic UV, Penic M, Brncic M, Bosiljkov T and Levaj B (2013). The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in Dalmatian wild sage (*Salvia officinalis* L.) extracts. *Food technology and biotechnology,* 51**:**84.

Diego-Taboada A, Beckett ST, Atkin SL and Mackenzie G (2014). Hollow pollen shells to enhance drug delivery. *Pharmaceutics,* 6**:**80-96.

Erdtman G (1960). The acetolysis method. A revised description. *Svensk Bot. Tidsk,* 54**:**561-4.

Escuredo O, Míguez M, Fernández-González M and Seijo MC (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food chemistry,* 138**:**851-56.

Feas X, Vazquez-Tato MP, Estevinho L, Seijas JA and Iglesias A (2012). Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules,* 17**:**8359-77.

Fernandez GE, Carvajal LI and Perez GA (2009). In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. *Nutrition Research,* 29**:**751-60.

Gilda S, Kanitkar M, Bhonde R and Paradkar A (2010). Activity of water-soluble turmeric extract using hydrophilic excipients. *LWT-Food Science and Technology,* 43**:**59-66.

Guideline Ich 1996. Q2 (R1) (2005) Validation of analytical procedures: text and methodology. *International conference on harmonization.*

Hagl S, Kocher A, Schiborr C, Eckert SH, Ciobanu I, Birringer M, El-Askary H, Helal A, Khayyal MT and Frank J (2013). Rice bran extract protects from mitochondrial dysfunction in guinea pig brains. *Pharmacological Research,* 76**:**17-27.

Haleagrahara N, Radhakrishnan A, Lee N and Kumar P (2009). Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats. *European journal of pharmacology,* 621**:**46-52.

Holcapek M, Lisa M, Jandera P and Kabatova N (2005). Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. *Journal of separation science,* 28**:**1315-33.

Hosseinzadeh R, Khorsandi K and Hemmaty S (2013). Study of the effect of surfactants on extraction and determination of polyphenolic compounds and antioxidant capacity of fruits extracts.

Jain S, Jain AK, Pohekar M and Thanki K (2013). Novel self-emulsifying formulation of quercetin for improved in vivo antioxidant potential: Implications for drug-induced cardiotoxicity and nephrotoxicity. *Free Radical Biology and Medicine,* 65**:**117-30.

Kandhare AD, Raygude KS, Ghosh P, Ghule AE and Bodhankar SL (2012). Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy. *Fitoterapia,* 83**:**650-59.

Kandhare AD, Shivakumar V, Rajmane A, Ghosh P and Bodhankar SL (2014). Evaluation of the neuroprotective effect of chrysin via modulation of endogenous biomarkers in a rat model of spinal cord injury. *Journal of natural medicines,* 68**:**586-603.

Ketkar S, Gopu C, Badgujar L, Paradkar A and Mahadik K (2011). Male sexual behavior improving effect of lipid based extract of Mucuna Pruriens in rats. *Pharmacologyonline,* 1**:**1-14.

Kiens B and Richter EA (1998). Utilization of skeletal muscle triacylglycerol during postexercise recovery in humans. *American Journal of Physiology-Endocrinology And Metabolism,* 275**:**E332-E37.

King TE (1967). Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. *Methods in Enzymology,* 10**:**322-31.

King TE and Howard RL (1962). The preparation and some properties of a reduced diphosphopyridine nucleotide dehydrogenase from the snake venom digest of a heart muscle preparation. *Journal of Biological Chemistry,* 237**:**1686-98.

Kubina RW, Kabała DA, Dziedzic A, Bielec B, Wojtyczka RD, Bułdak RJ, Wyszyńska M, Stawiarska PB and Szaflarska SE (2015). The Ethanol Extract of Polish Propolis Exhibits Anti-Proliferative and/or Pro-Apoptotic Effect on HCT 116 Colon Cancer and Me 45 Malignant Melanoma Cells *In Vitro* Conditions. *Advances in Clinical and Experimental Medicine,* 24**:**203-12.

Kumar A, Prakash A and Dogra S (2011). Centella asiatica attenuates D-galactose-induced cognitive impairment, oxidative and mitochondrial dysfunction in mice. *International journal of Alzheimer's disease,* 2011.

Kumazawa S, Taniguchi M, Suzuki Y, Shimura M, Kwon M-S and Nakayama T (2002). Antioxidant activity of polyphenols in carob pods. *Journal of agricultural and food chemistry,* 50**:**373-77.

Lee SJ (2004). Regulation of muscle mass by myostatin. *Annu. Rev. Cell Dev. Biol.,* 20**:**61-86.

Lee SJ (2007). Sprinting without myostatin: a genetic determinant of athletic prowess. *Trends in genetics,* 23**:**475-77.

Leja M, Mareczek A, Wyżgolik G, Klepacz-Baniak J and Czekońska K (2007). Antioxidative properties of bee pollen in selected plant species. *Food Chemistry,* 100**:**237-40.

Lenk K, Schur R, Linke A, Erbs S, Matsumoto Y, Adams V and Schuler G (2009). Impact of exercise training on myostatin expression in the myocardium and skeletal muscle in a chronic heart failure model. *European journal of heart failure,* 11**:**342-48.

Liu Y, Peterson DA, Kimura H and Schubert D (1997). Mechanism of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction. *Journal of neurochemistry,* 69**:**581-93.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J biol Chem,* 193**:**265-75.

Maack A (2007). Soluble composition containing sporopollenin andthe use thereof. *U.S. Patent No. 7,182,965*.

Marghitas LA, Stanciu OG, Dezmirean DS, Bobis O, Popescu O, Bogdanov S and Campos MG (2009). In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chemistry,* 115**:**878-83.

Marinova D, Ribarova F and Atanassova M (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *journal of the university of chemical technology and metallurgy,* 40**:**255-60.

Matsakas A, Bozzo C, Cacciani N, Caliaro F, Reggiani C, Mascarello F and Patruno M (2006). Effect of swimming on myostatin expression in white and red

gastrocnemius muscle and in cardiac muscle of rats. *Experimental physiology,* 91**:**983-94.

Matsakas A, Friedel A, Hertrampf T and Diel P (2005). Short-term endurance training results in a muscle-specific decrease of myostatin mRNA content in the rat. *Acta physiologica scandinavica,* 183**:**299-307.

Merrill AL and Watt BK (1955). Energy value of foods-basis and derivation. *Energy value of foods-basis and derivation.*

Miranda KM, Espey MG and Wink DA (2001). A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric oxide,* 5**:**62-71.

Misra HP and Fridovich I (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological chemistry,* 247**:**3170-75.

Mondal AK, Parui S and Mandal S (1998). Biochemical analysis of four species of Cassia L. pollen. *Aerobiologia,* 14**:**45-50.

Moron MS, Depierre JW and Mannervik B (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 582**:**67-78.

Official Methods of Analysis of AOAC International (2005). Soxhlet extraction method. *Association of Official Analytical Chemist.***:**Ref. 963.15.

Official Methods of Analysis of AOAC International (2006). Microchemical determination of nitrogen. Micro-Kjeldahl method. *Association of Official Analytical Chemist.***:**Ref. 960.52.

Palstra AP, Rovira M, Rizo D, Torrella JR, Spaink HP and Planas JV (2014). Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. *BMC genomics,* 15**:**1136.

Pouton CW (2000). Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying'drug delivery systems. *European Journal of Pharmaceutical Sciences,* 11**:**S93-S98.

Powers SK and Jackson MJ (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiological reviews,* 88**:**1243-76.

Prajapati HN, Dalrymple DM and Serajuddin AT (2012). A comparative evaluation of mono-, di-and triglyceride of medium chain fatty acids by lipid/surfactant/water phase diagram, solubility determination and dispersion testing for application in pharmaceutical dosage form development. *Pharmaceutical research,* 29**:**285-305.

Rajasekaran A, Sivagnanam G and Xavier R (2008). Nutraceuticals as therapeutic agents: A Review. *Res J Pharm Tech,* 1**:**328-40.

Rosenthal RE, Hamud F, Fiskum G, Varghese PJ and Sharpe S (1987). Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *Journal of Cerebral Blood Flow and Metabolism,* 7**:**752-58.

Roulston T and Cane JH (2000). Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution,* 222**:**187-209.

Sathiyanarayanan L, Paradkar AR and Mahadik KR (2010). In vivo and in vitro antioxidant activity of lipid based extract of Bacopa monniera Linn. compared

to conventional extract and traditional preparation. *European Journal of Integrative Medicine,* 2**:**93-101.

Schakel SF, Buzzard IM and Gebhardt SE (1997). Procedures for estimating nutrient values for food composition databases. *Journal of Food Composition and Analysis,* 10**:**102-14.

Schneider CD and Oliveira ARD (2004). Oxygen free radicals and exercise: mechanisms of synthesis and adaptation to the physical training. *Revista Brasileira de Medicina do Esporte,* 10**:**308-13.

Singleton V, Orthofer R and Lamuela-Raventos R (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology,* 299**:**152-78.

Slater T and Sawyer B (1971). The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro. General features of the systems used. *Biochem. J,* 123**:**805-14.

Smith RC and Lin BK (2013). Myostatin inhibitors as therapies for muscle wasting associated with cancer and other disorders. *Current opinion in supportive and palliative care,* 7**:**352.

Sottocasa GL, Kuylenstierna B, Ernster L and Bergstrand A (1967). An electron-transport system associated with the outer membrane of liver mitochondria a biochemical and morphological study. *The Journal of Cell Biology,* 32**:**415-38.

Sriram S, Subramanian S, Sathiakumar D, Venkatesh R, Salerno MS, Mcfarlane CD, Kambadur R and Sharma M (2011). Modulation of reactive oxygen species in skeletal muscle by myostatin is mediated through NF-κB. *Aging cell,* 10**:**931-48.

Surapaneni DK, Adapa SRSS, Preeti K, Teja GR, Veeraragavan M and Krishnamurthy S (2012). Shilajit attenuates behavioral symptoms of chronic fatigue syndrome by modulating the hypothalamic–pituitary–adrenal axis and mitochondrial bioenergetics in rats. *Journal of ethnopharmacology,* 143**:**91-99.

Swellam T, Miyanaga N, Onozawa M, Hattori K, Kawai K, Shimazui T and Akaza H (2003). Antineoplastic activity of honey in an experimental bladder cancer implantation model: in vivo and in vitro studies. *International journal of urology,* 10**:**213-19.

Szczesna T (2006a). Long-chain fatty acids composition of honeybee-collected pollen. *Journal of Apicultural Science,* 50**:**65-79.

Szczesna T (2006b). Protein content and amino acid composition of bee-collected pollen from selected botanical origins. *J apic Sci,* 50**:**81-90.

Tagliazucchi D, Verzelloni E, Bertolini D and Conte A (2010). In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry,* 120**:**599-606.

Thirumalai T, Therasa SV, Elumalai E and David E (2011). Intense and exhaustive exercise induce oxidative stress in skeletal muscle. *Asian Pacific Journal of Tropical Disease,* 1**:**63-66.

Tomas BFA, Tomas LF, Ferreres F and Garcia Viguera C (1989). Flavonoids as biochemical markers of the plant origin of bee pollen. *Journal of the Science of Food and Agriculture,* 47**:**337-40.

Wehling M, Cai B and Tidball JG (2000). Modulation of myostatin expression during modified muscle use. *The FASEB Journal,* 14**:**103-10.

Westerblad H, Allen DG and Lännergren J (2002). Muscle fatigue: lactic acid or inorganic phosphate the major cause? *Physiology,* 17**:**17-21.

Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P and Knight A (2003). Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochemical and biophysical research communications,* 300**:**965-71.

Willoughby DS (2004). Effects of heavy resistance training on myostatin mRNA and protein expression. *Medicine and science in sports and exercise,* 36**:**574-82.

Woisky RG and Salatino A (1998). Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of apicultural research,* 37**:**99-105.

Yazdi AS (2011). Surfactant-based extraction methods. *TrAC Trends in Analytical Chemistry,* 30**:**918-29.

Chapter 5. PROPOLIS: *Exploration of Green Extraction Method*

This chapter will cover studies performed on propolis. Part A of the chapter includes development and validation of HPLC-PDA method for simultaneous estimation of flavonoid components of propolis while Part B includes microwave assisted extraction (MAE) of propolis using Gelucire 44/14 as exractant in comparison with conventional organic solvent. This is followed by studies on dielectric properties of Gelucire 44/14 solutions determining the mechanism of Gelucire 44/14 based MAE.

5A. Development of HPLC-PDA method for simultaneous estimation of principal flavonoid marker components of propolis

5A.1. Experimental work:

5A.1.1. Preparation of standard solution

Individual standard stock solutions of flavonoid markers caffeic acid, rutin, quercetin, apigenin, kaempferol, chrysin, acacetin, galangin and caffeic acid phenethyl ester (CAPE) (each 1 mg/ml) were prepared in methanol. The solutions were diluted with methanol to obtain mixed working standard solutions of concentration 5, 10, 20, 40, 60 and 80 µg/ml each.

5A1.2. HPLC-PDA conditions:

HPLC-PDA analysis was performed on Jasco HPLC system (Tokyo, Japan) using Thermo-Hypersil GOLD C18 RP column (250 mm ×4.0 mm, 5 µm) coupled with Thermo-Hypersil BDS C-18 guard column (30 mm × 4.6 mm, 5 µm). The system was equipped with Jasco PU-2089 Plus quaternary gradient pump, Jasco multiwavelength detector (PDA, MD-2010Plus) and ChromPass software (Version 1.8.6.1). Samples were injected through Rheodyne injector with 20 µL loop. The mobile phase comprised water adjusted to pH 3.0 with formic acid (solvent A) and acetonitrile (solvent B) mixed using a linear gradient system; initial 10% B, 10%-20% B in 3 min, held constant till 10 min, 20-25% B in 20 min, 25-40% B in 45 min, held constant till 50 min. Solvent B was decreased to 10% B till 52 min to reach initial conditions and held for next 13 min for preconditioning. The elution was performed at a flow rate of 1 ml/min at ambient temperature. The PDA acquisitions were performed between 200 to 400 nm and detection wavelengths were set at 265 nm (for chrysin, galangin); 328 nm (for caffeic acid, Cape); 336 nm (for apigenin, acacetin); 356 nm (for rutin) and 370 nm (for quercetin, kaempferol). [Characteristic wavelength of maximum absorption (λmax) for respective compounds]. Each sample was analyzed in triplicate injections. Calibration curves were plotted for the standard solutions.

5A.1.3. Method validation

The optimized HPLC-PDA method was validated based on following parameters as per ICH guidelines (Guideline ICH, 1996).

5A.1.3.1. Linearity and range:

Linearity of the method was studied by injecting the mixed working standard solutions of concentrations 5, 10, 20, 40, 60 and 80 µg/ml each in triplicate into the HPLC system. The peak area was recorded for all the individual flavonoid markers and calibration graph for each compound was obtained by plotting peak area versus concentration at each level.

5A.1.3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is the smallest concentration of the analyte that gives a measurable response. LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified. The LOD and LOQ were determined for each compound by calibration curve method using standard deviation (SD) and slope (S) of the calibration curve for each compound as follows.

$$LOD = \frac{3.3 \times Sy.x}{S}; \quad LOQ = \frac{10.0 \times Sy.x}{S}$$

Where, Sy.x is Standard deviation of residuals from line; S is slope.

5A.1.3.3. Precision

The precision of system was ascertained by intra-day (repeatability) and inter-day (intermediate precision) injections at three different concentrations in triplicates. The repeatability studies were performed by injecting three different concentrations 5, 40 and 80 µg/ml of the mixed working standard solution three times on the same day and recording the corresponding peak areas. Intermediate precision studies were performed by analysis of the different concentrations 5, 40 and 80 µg/ml, three times on three different days. Relative standard deviation (% RSD) was determined to check the repeatability of sample application and measurement of peak areas.

5A.1.3.4. Specificity

Chromatograms of sample, standard and blank solutions were qualitatively compared and the peak purity values as detected by PDA detector for standards and sample solution were ascertained in order to ensure the specificity of method.

5A.1.3.5. Accuracy

Standard addition method was employed to determine percentage recovery of the flavonoid compounds from extract sample. Prior to the addition of known quantities of standard markers, the background levels of individual flavonoids in extract sample were determined in triplicates.

5A.1.3.1.6. Robustness of the method

Robustness of the method was determined by making slight changes in the chromatographic conditions in terms of flow rate, pH of mobile phase and analytical columns. Robustness of the method was ascertained at three different concentration levels viz. 5, 40 and 80 µg/ml for individual marker compounds.

5A.2. Results and Discussion:

HPLC-PDA method optimization and validation:

Based on the reported methods (Gardana et al., 2007, Pellati et al., 2013) mobile phase comprising of water adjusted to pH 3.0 with Formic acid (solvent A) and acetonitrile (solvent B) was adopted for analysis on the HPLC-PDA system. Linear gradient for mixing of the solvents was optimized to achieve resolution of the analytes with stable baseline. Figure 5.1.depicts stacked view of representative chromatogram for the standard solution extracted at respective wavelengths of detection for each standard marker.

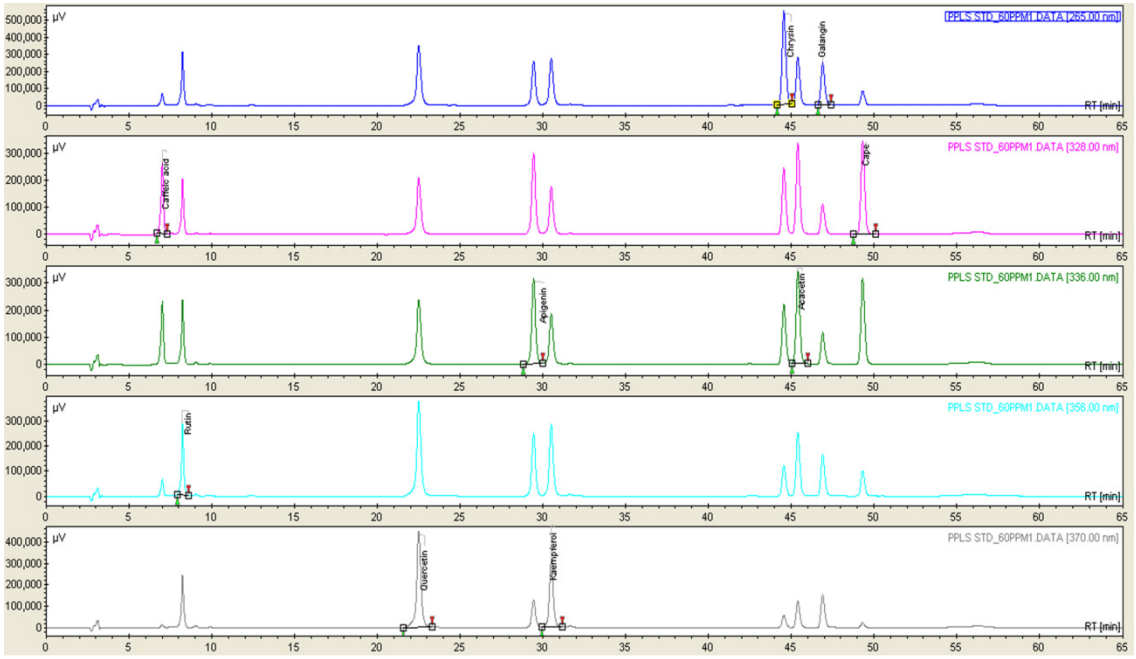


Figure 5.1. Stacked view of representative chromatogram for the standard solution extracted at respective wavelengths of detection for each standard marker

The chromatogram illustrated well resolved flavonoid marker compounds within 65 min run. The Rt values (min ± SD) observed for standard markers are as follows: caffeic acid (7.01 ± 0.02), rutin (8.22 ± 0.01), quercetin (22.48 ± 0.12), apigenin (29.42 ± 0.02), kaempferol (30.49 ± 0.03), chrysin (44.54 ± 0.01), acacetin (45.39 ± 0.02), galangin (46.89 ± 0.11) and cape (49.31 ± 0.02).

Figure 5.2 (A-I) depicts UV spectra for the standards recorded with PDA multiwavelength detector.

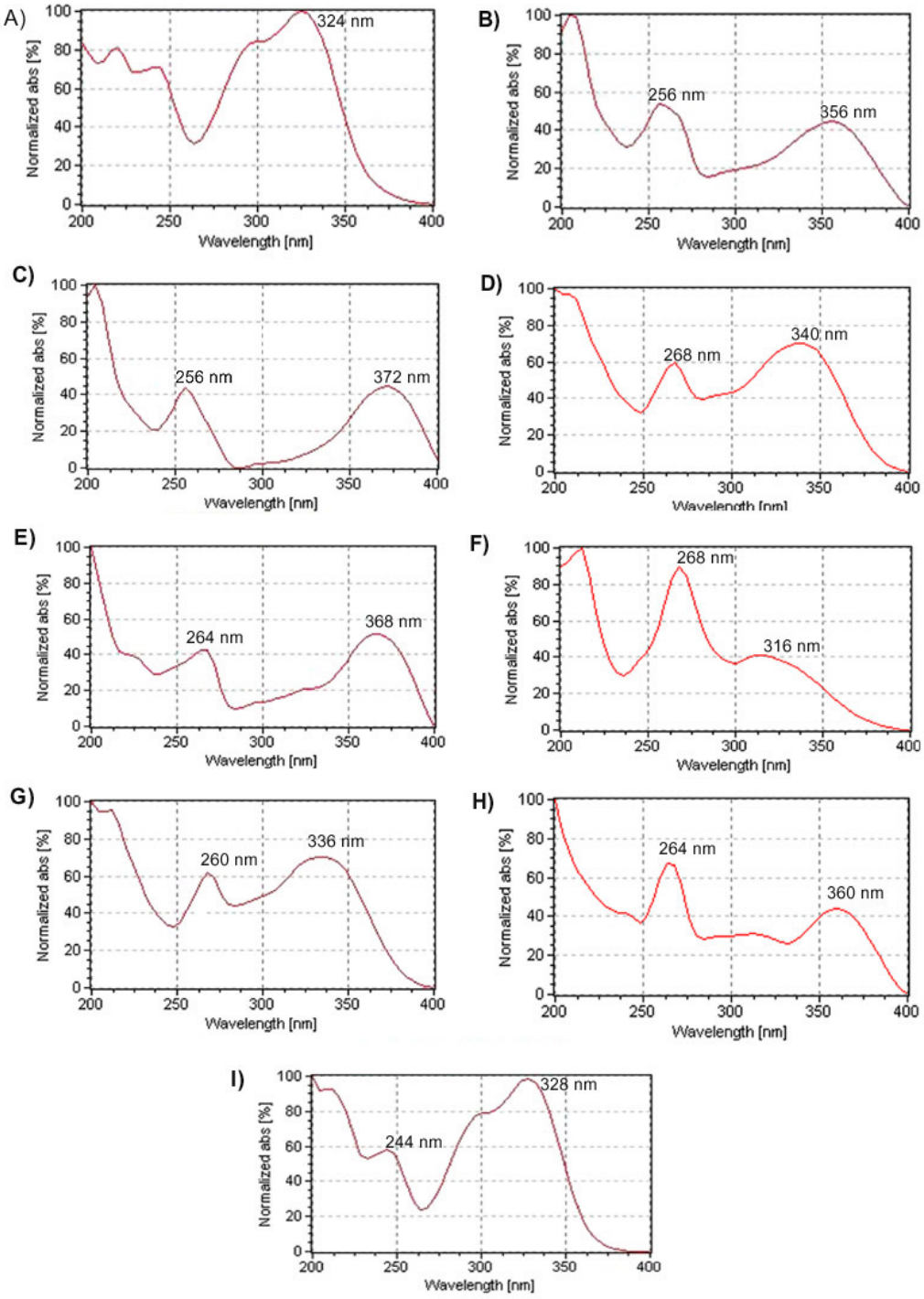


Figure 5.2. UV spectra for standards (A) caffeic acid, (B) Rutin, (C) Quercetin, (D) Apigenin, E) Kaempferol, F) Chrysin, G) Acacetin, H) Galangin, I) Caffeic acid phenethyl ester (CAPE)

Results for validation parameters with ANOVA and residual analysis are summarized in Table 5.1.

Linearity:

The HPLC-PDA method was validated and showed good linearity (r^2> 0.999) in the concentration range of 5-80 µg/ml to quantify the constituents in the sample solutions. The calibration curve parameters for the screened markers showed linear relationship between peak area and concentration for each compound. Figure 5.3 A-I demonstrated linear calibration curves for individual marker compounds. Values of the slope and intercept for each compound are mentioned in table 5.1.

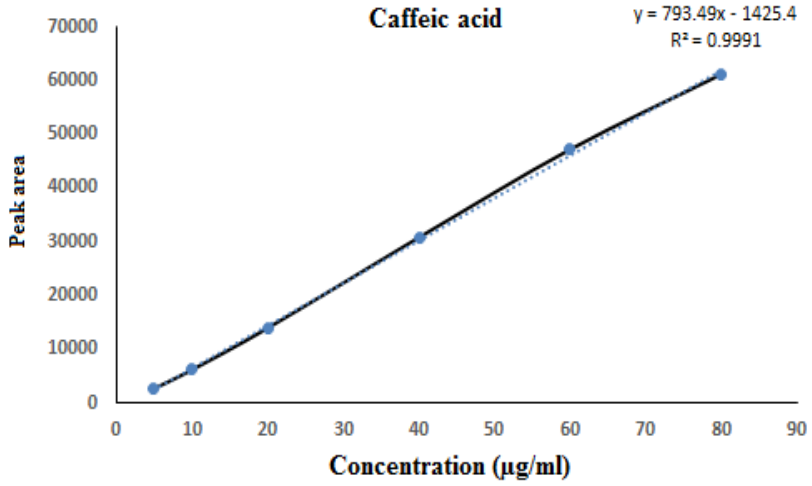


Figure 5.3A. Linear calibration curve of caffeic acid

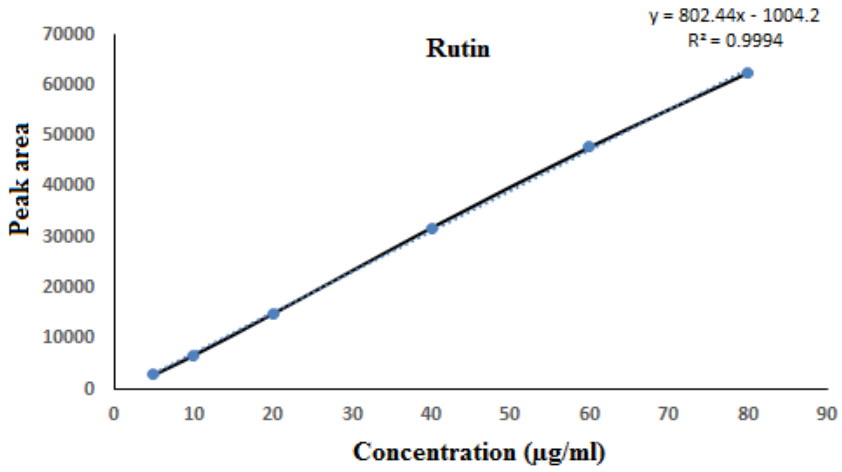


Figure 5.3B. Linear calibration curve of Rutin

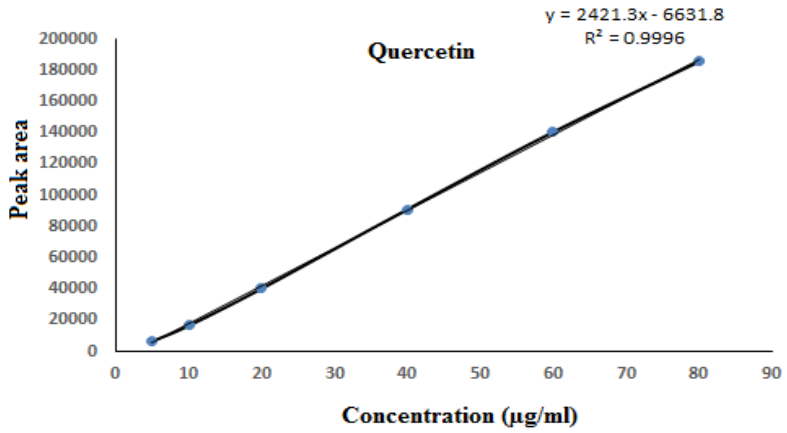


Figure 5.3C. Linear calibration curve of quercetin

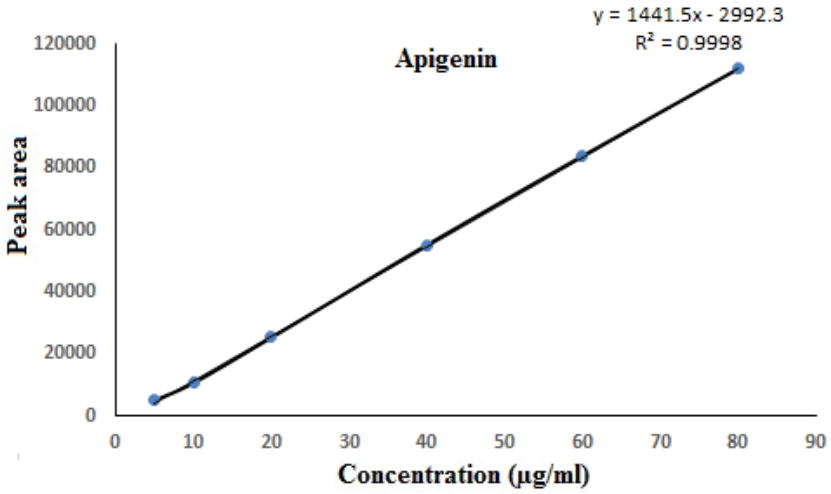


Figure 5.3D. Linear calibration curve of apigenin

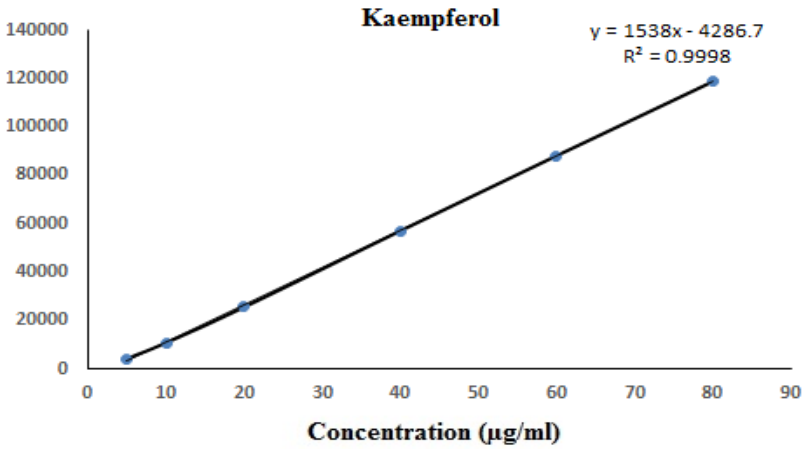


Figure 5.3E. Linear calibration curve of kaempferol

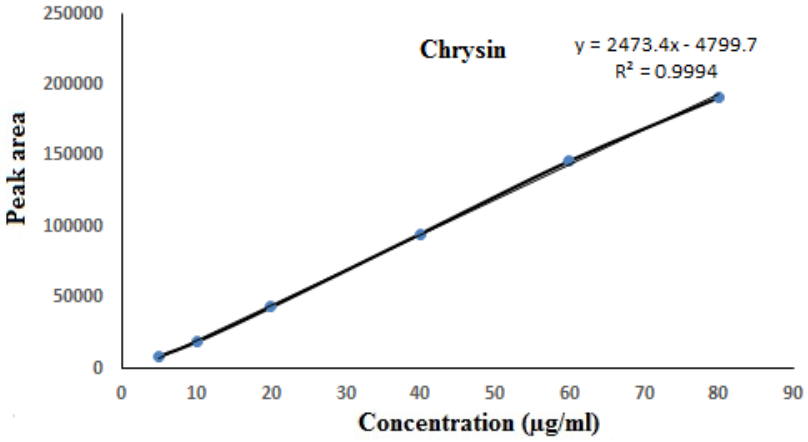


Figure 5.3F. Linear calibration curve of chrysin

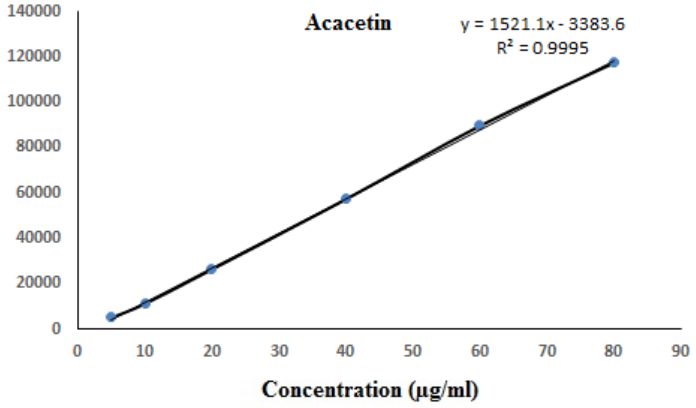


Figure 5.3G. Linear calibration curve of acacetin

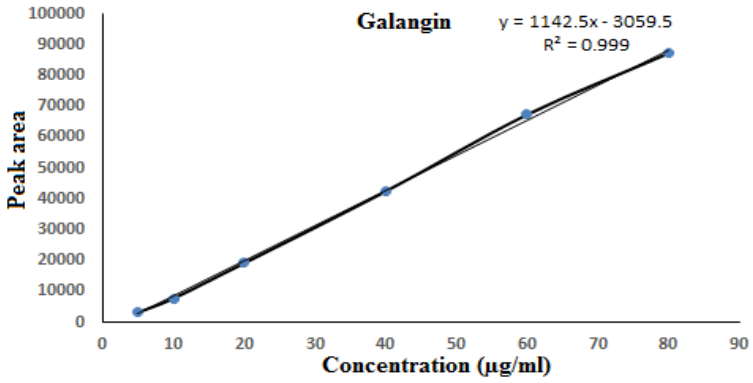


Figure 5.3H. Linear calibration curve of galangin

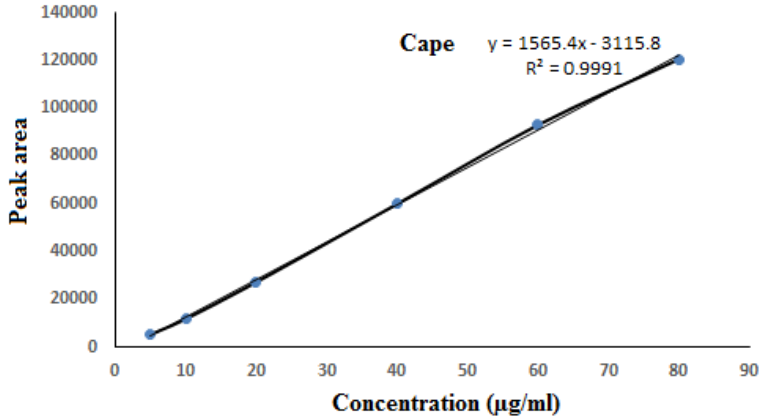


Figure 5.3I. Linear calibration curve of cape

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ values obtained for the respective marker compounds are displayed in table 5.1.

Table 5.1: Results of validation parameters including statistical data for calibration curves (n=3)

Analytes	Regression Equation	r^2	Linear range ug/ml	Slope (mean±SEM)	Intercept (mean±SEM)	Confidence limit of slope (95 % CI)	Confidence limit of intercept (95 % CI)	Sy.x (standard deviation of residuals from		P value	Specificity	LOD ug/ml	LOQ ug/ml
								y(i.e., area) on x(i.e., concentration)					
Caffeic acid	y = 793.49x - 1425.4	0.9991	5--80	793.5 ± 11.64	-1425 ± 523.1	761.2 to 825.8	-2877 to 26.66	371.24		< 0.0001	Specific	1.543	4.678
Rutin	y = 802.44x - 1004.2	0.9994	5--80	802.4 ± 9.682	-1004 ± 435.3	775.6 to 829.3	-2212 to 204.1	347.50		< 0.0001	Specific	1.429	4.330
Quercetin	y = 2421.3x - 6631.8	0.9996	5--80	2421 ± 24.82	-6632 ± 1116	2352 to 2490	-9729 to -3535	876.00		< 0.0001	Specific	1.194	3.618
Apigenin	y = 1441.5x - 2992.3	0.9998	5--80	1441 ± 10.99	-2992 ± 493.8	1411 to 1472	-4363 to -1621	575.99		< 0.0001	Specific	1.319	3.997
Kaempferol	y = 1538x - 4286.7	0.9998	5--80	1538 ± 9.922	-4287 ± 446.0	1510 to 1566	-5525 to -3049	468.22		< 0.0001	Specific	1.004	3.044
Chrysin	y = 2473.4x - 4799.7	0.9994	5--80	2473 ± 30.68	-4800 ± 1379	2388 to 2559	-8628 to -970.8	222.85		< 0.0001	Specific	0.297	0.901
Acacetin	y = 1521.1x - 3383.6	0.9995	5--80	1521 ± 17.33	-3384 ± 778.9	1473 to 1569	-5546 to -1221	237.56		< 0.0001	Specific	0.515	1.561
Galangin	y = 1142.5x - 3059.5	0.9990	5--80	1142 ± 18.01	-3060 ± 809.8	1092 to 1192	-5308 to -811.6	349.77		< 0.0001	Specific	1.010	3.062
CAPE	y = 1565.4x - 3115.8	0.9991	5--80	1565 ± 22.91	-3116 ± 1030	1502 to 1629	-5974 to -257.4	387.68		< 0.0001	Specific	0.817	2.477

*P value <0.000 1 is considered extremely significant. SEM: standard error of mean

Precision

Table 5.1.1 displays % RSD values for all standards at respective concentrations of 5, 40 and 80 µg/ml. Calculated % RSD values for all the standards were observed in the range of 0.026 to 1.988 indicating acceptable intra-day and inter-day variation with respect to working standards.

Table 5.1.1. Intra-day and inter-day precision data of HPLC-PDA method for flavonoid markers (n=3)

Standard	Conc (µg/ml)	Repeatability (intra-day)			Intermediate precision (inter-day)		
		Found conc ± SD	% RSD	SE	Found conc ± SD	% RSD	SE
Caffeic acid	5	4.983± 0.077	1.558	0.025	5.016± 0.051	1.022	0.017
	40	40.216± 0.557	1.385	0.185	40.396± 0.618	1.53	0.206
	80	79.490± 0.972	1.223	0.324	80.32± 0.683	0.851	0.227
Rutin	5	5.063± 0.047	0.933	0.015	5.046± 0.075	1.5	0.025
	40	39.926± 0.255	0.639	0.085	39.920± 0.599	1.501	0.199
	80	80.253± 0.735	0.916	0.245	79.833± 1.365	1.709	0.455
Quercetin	5	4.976± 0.096	1.930	0.032	4.956± 0.085	1.715	0.028
	40	40.403± 0.751	1.861	0.250	40.380± 0.399	0.989	0.133
	80	79.333± 1.537	1.937	0.512	79.166± 1.530	1.933	0.510
Apigenin	5	5.05± 0.065	1.298	0.021	5.036± 0.076	1.516	0.025
	40	40.446± 0.630	1.557	0.21	40.183± 0.796	1.982	0.265
	80	80.2± 0.655	0.817	0.218	80.133± 0.503	0.628	0.167
Kaempferol	5	5.036± 0.100	1.988	0.033	4.980± 0.087	1.751	0.029
	40	40.276± 0.740	1.837	0.246	40.2± 0.360	0.896	0.120

	80	80.096± 0.894	1.116	0.298	80.073± 0.797	0.996	0.265
Chrysin	5	5.02± 0.026	0.527	0.008	5.006± 0.015	0.305	0.005
	40	40.023± 0.041	0.104	0.013	40.02± 0.036	0.09	0.012
	80	80.01± 0.05	0.062	0.016	80.00± 0.020	0.026	0.006
Acacetin	5	4.996± 0.032	0.643	0.01	5.016± 0.032	0.64	0.01
	40	40.016± 0.055	0.137	0.018	39.99± 0.03	0.075	0.01
	80	79.67± 0.603	0.761	0.202	80.00± 0.102	0.128	0.034
Galangin	5	4.957± 0.071	1.437	0.023	4.963± 0.087	1.760	0.029
	40	39.953± 0.058	0.146	0.019	39.813± 0.321	0.808	0.107
	80	79.913 ± 0.244	0.305	0.081	80.00± 0.275	0.344	0.091
Cape	5	4.973± 0.075	1.522	0.025	4.98± 0.07	1.405	0.023
	40	39.906± 0.112	0.281	0.037	40.323± 0.612	1.517	0.204
	80	79.73± 1.178	1.478	0.392	80.273± 0.652	0.813	0.217

Specificity:

No peak interference for standards, sample and blank solutions at respective retention times indicated specificity of method as depicted in figures 5.1, 5.5, 5.6. Peak purity values for flavonoid peaks were always greater than 998.607 for standard and sample solutions indicating absence of additional co-eluting peaks.

Accuracy:

Obtained results of recovery experiment are mentioned in table 5.1.2. The accuracy in terms of recovery percentage with small % RSD in the range of 96.802 % to 102.416 % indicates reliability of the method for quantification of the screened flavonoids.

Table 5.1.2.Recovery studies of HPLC-PDA method for flavonoid markers (n=3)

Flavonoid Screened	Added concentration (µg/ml)	Measured concentration (µg/ml)	% Recovery	% RSD
Caffeic acid				
10.629	10	19.886	96.398	1.281
10.416	20	29.941	98.438	1.36
10.711	30	39.486	96.990	1.512
Quercetin				
11.225	10	20.871	98.332	1.681
10.682	20	31.126	101.447	0.894
10.206	30	40.446	100.596	1.962
Apigenin				
10.049	10	19.686	98.189	1.336
10.188	20	30.148	99.867	1.49
10.221	30	40.632	101.021	1.293
Kaempferol				
24.046	10	34.14	100.276	2.011
24.664	20	43.871	98.224	1.231
24.028	30	54.466	100.810	1.462
Chrysin				
66.847	10	66.221	99.063	1.718
67.221	20	66.320	98.659	1.886
66.554	30	67.116	100.844	1.924
Acacetin				
5.024	10	22.126	97.764	0.819
5.745	20	31.882	99.117	1.126
5.307	30	41.648	98.022	0.487
Galangin				
39.872	10	49.666	99.586	0.672
40.536	20	59.632	101.394	0.801
39.697	30	68.469	99.006	0.422
Cape				
32.732	10	42.446	99.330	1.214
31.446	20	50.821	98.785	1.468
32.668	30	62.722	100.086	1.128

Robustness:

The factor flow rate was varied at three levels (−1, 0 and +1) viz. 0.9, 1 and 1.1 ml/min. The two analytical columns, Hypersil GOLD C18 column by Thermo Scientific, USA and HiQ-Sil™ HS C18 column by Kromatek, Japan, were employed during the experiment. pH of mobile phase was varied as 2.5, 3 and 3.5. One factor at a time was changed to estimate the effect. The replicate injections (n = 3) of mixed standard solution at three concentration levels were performed under the changes of chromatographic parameters (factors). No marked changes in chromatograms were observed upon the deliberate changes made in parameters flow rate, pH of mobile phase and type of column indicating the robustness of developed method.

5A3. Conclusion:

The HPLC-PDA method for simultaneous estimation of principal flavonoid marker components of propolis was developed and validated as per ICH guidelines. Accurate quantification of individual marker components was ensured through PDA by analyzing the components at respective wavelength of maximum absorption (λmax). The method was evaluated for linearity, precision, specificity, accuracy and robustness so as to ascertain the suitability of method for analysis of extracts. The method was found to be selective and linear between concentration range of 5 to 80 µg/ml. Statistical analysis proved that the method is suitable for simultaneous analysis of caffeic acid, rutin, quercetin, apigenin, kaempferol, chrysin, acacetin, galangin and CAPE from propolis sample.

5B. Microwave Assisted Extraction of Propolis:

5B.1. Instrumentation for Microwave Assisted Extraction:

A monowave focused closed-vessel Discover system 908010 (CEM, Matthews INC, USA) with programmable conditions of temperature and time was employed in the study (Figure 5.4). The system offered magnetron frequency of 2.45 GHz, extended operation limit up to temperature of 300 °C and pressure of 30 bar (435 psi) open up. The microwave power (maximum up to 300 W) and pressure were automatically adjusted by internal temperature sensing system and integrated hydraulic pressure sensor. The Discover system was capable of supplying a continuous non-pulse microwave heating by focusing on a single cavity wherein the sample was placed. A high-performance magnetic stirring device provided agitation and homogenization required during the extraction process.

Figure 5.4. Monowave focused closed-vessel Discover system 908010 (CEM, Matthews INC, USA)

5B.1.1. Conventional microwave assisted extraction of propolis:

5B.1.1.2. Optimization of MAE conditions:

i. Finely ground propolis (0.5 g each) was mixed with 5 ml of ethanol in 30 ml capacity glass tubes.
ii. The mixtures were individually subjected to microwave irradiation at different conditions of extraction temperature and time under continuous stirring at 600

rpm (Table 5.2). Upon completion of extraction time, the glass tube was allowed to cool at room temperature before opening the lid.

iii. Individually the extracts were centrifuged for 5 min at 4000 rpm using Allegra Centrifuge, supernatants were filtered through 0.45 µ membrane filter. The extract samples were diluted with methanol (10X) and subjected to HPLC-PDA analysis for determination of flavonoid compounds.

The extraction conditions were optimized based on the maximum amount of total flavonoids content in terms of screened markers observed in the extracts.

Table 5.2. Different experimental conditions screened for conventional MAE of propolis

No.	Batch code	Temperature (± 0.5 °C)	Time (min)
1	MW-EEP 45_10	45	10
2	MW-EEP 45_20	45	20
3	MW-EEP 65_10	65	10
4	MW-EEP 65_20	65	20
5	MW-EEP 105_10	105	10

5B.1.2. Gelucire 44/14 based MAE of propolis:

i. Different concentrations (10, 20, 30, 40 and 50% w/w) of Gelucire 44/14 aqueous solutions were prepared by dissolving respective amount of Gelucire 44/14 into distilled water.

ii. Finely ground propolis (0.5 g) was mixed individually with 5 ml of each solution in glass tubes.

iii. Individually the mixtures were subjected to MAE at the optimized conditions of 65 ±0.5°C for 20 min under continuous stirring at 600 rpm to produce Gelucire 44/14 extracts of propolis (MW-GP10, MW-GP20, MW-GP30, MW-GP40 and MW-GP50 respectively). The glass tubes were allowed to cool at room temperature before opening.

iv. Individually each of the extract was centrifuged for 5 min at 4000 rpm using Allegra Centrifuge, supernatant was filtered through 0.45 membrane filter, diluted with methanol (10X) and subjected to HPLC-PDA analysis for determination of flavonoid compounds.

Similarly an aqueous extract of propolis (MW-DWP) was prepared using distilled water as solvent for comparison.

5B.2. Determination of dielectric properties of Gelucire 44/14 solutions

Dielectric properties for Gelucire 44/14 aqueous solutions were measured using 85070B open ended coaxial line probe connected to E5071C vector network analyzer (Agilent Technologies, Malaysia). Dielectric constant and loss were calculated by 85070D dielectric probe kit software (Agilent Technologies) based on the reflection coefficient of the substance in contact with the tip of probe. Frequency was set at a range of 300 kHz to 2.45GHz wherein most of the industrial microwave systems operate (Datta, 2001). Measurements were made at 25; 45 and 65 (± 0.5) °C.

Procedure:

i. Each beaker of 10 ml size containing individual solution was placed on the platform of 50 mm diameter.
ii. The downward open-ended coaxial-line probe was immersed completely in the solvent. At most care was taken to avoid air bubbles between the solvent and the probe surface. Three replications per sample were performed.
iii. Values for dielectric constant and loss factor at 2.45GHz were recorded to determine the dissipation factor or loss tangent 'Tan δ'.

5B.3. Differential scanning calorimertry:

Subzero calorimetric measurements for aqueous Gelucire 44/14 solutions were performed with Mettler Toledo 821e instrument equipped with an intracooler (Mettler Toledo, Switzerland).

Procedure:

Individually 15 ±3 mg of Gelucire 44/14 solution of each concentration (10, 20, 30, 40 and 50% w/w) was placed in closed aluminum crucibles and cooled to -30 °C at the rate of 10 °C/min. The samples were maintained at -30 °C for 10 min and subjected to heating cycle from -30 °C to 10 °C at the scanning rate of 3 °C/min. To ensure the accuracy of caloric data, the instrument was calibrated with Indium/zinc.

5B.4. Results and Discussion

Determination of flavonoids from propolis extracts obtained by conventional MAE:

Table 5.3. Flavonoids content (mg/g) for conventional microwave extracts (MW-EEP) at different conditions analyzed:

Flavonoid compounds	MW-EEP 45_10 (45°C/10 min)	MW-EEP 45_20 (45°C/ 20 min)	MW-EEP 65_10 (65°C/ 10 min)	MW-EEP 65_20 (65°C/ 20 min)	MW-EEP 105_10 (105°C/ 10 min)
Caffeic acid	0.651± 0.012	0.684 ± 0.064	0.742 ± 0.021	0.807 ± 0.037	BDL
Rutin	BDL	BDL	BDL	BDL	BDL
Quercetin	0.635 ± 0.017	0.696± 0.011	0.723 ± 0.04	0.787± 0.009	0.732 ± 0.019
Apigenin	0.567 ± 0.034	0.585 ± 0.004	0.616 ± 0.013	0.865 ± 0.164	0.599 ±0.003
Kaempferol	1.264 ± 0.006	1.180 ± 0.014	1.508 ± 0.003	2.337 ± 0.138	2.196 ± 0.018
Chrysin	5.524 ± 0.012	5.841 ± 0.018	6.736 ± 0.103	13.509 ±0.060	9.142 ± 0.297
Acacetin	1.01 ± 0.071	0.692 ± 0.036	0.921 ± 0.025	1.45 ± 0.156	0.690 ± 0.01
Galangin	13.956 ±0.061	17.083 ±0.003	23.52 ± 0.572	39.116 ± 2.00	30.681 ± 2.54
Cape	1.547 ± 0.055	1.626 ± 0.007	1.585 ± 0.002	3.323 ± 0.366	2.626 ± 0.21
Total flavonoids	25.114 ±0.153	28.390 ±0.042	36.353 ± 0.627	62.201 ±1.372**	45.356 ±0.994**

Data expressed as Mean ± SD (n=3);
BDL: Below detection limit; Data was analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison test (** P <0.001)

Table 5.3 depicts HPLC-PDA results of flavonoids content for MW-EEP prepared at different set of microwave conditions. Improvement in the extraction yield of total flavonoids was observed with increased temperature and duration of MAE. Significant increase ($p < 0.001$) in the yield of total flavonoids content in terms of screened markers was observed at 65°C/20 min. MAE at 105°C for 10 min demonstrated significant decline ($p < 0.01$) in the total flavonoids content. Based on the highest total flavonoids content (62.201 ± 1.372 mg/g) in terms of screened markers, the MAE conditions were

optimized to 65 ± 0.5 °C for 20 min. Figure 5.5 shows representative chromatogram for MW-EEP 65_20 extracted at respective wavelengths of detection for screened markers.

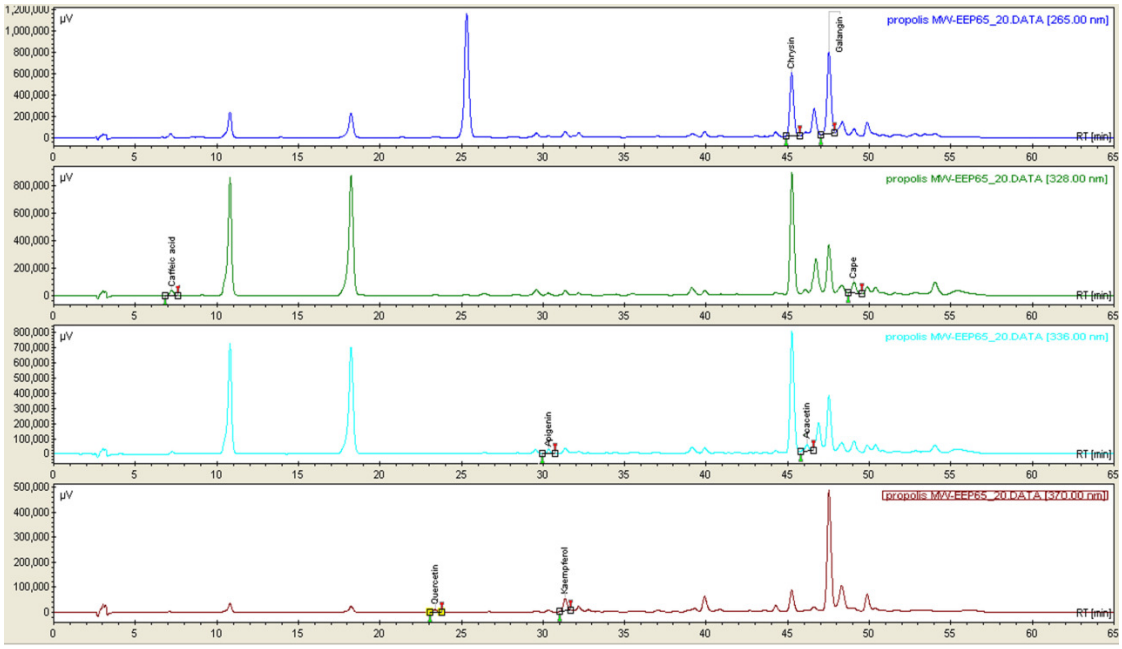


Figure 5.5. Stacked view of representative chromatogram for MW-EEP 65_20 extracted at respective wavelengths of detection for screened markers

For MAE especially in case of closed vessel assembly, process temperature, time, microwave irradiation power, type of solvent and drug to solvent ratio are the major factors which affect the extraction efficiency (Pellati et al., 2013). In order to optimize the operating conditions and reduce the variables to minimum, we have varied temperature and time of MAE at constant drug to solvent ratio of 1:10 (w/v). An organic solvent ethanol which is reported to be a good solvent for propolis flavonoid constituents was selected as an organic solvent for extraction (Trusheva et al., 2007). The closed-vessel MAE assembly employed herein regulates microwave irradiation power and pressure based on the selected temperature, hence could not be controlled during process. However the applied conditions did not exceed the microwave power above 150 W, hence maintained the low irradiation power throughout process. Under these set of conditions, extraction yield of total flavonoids was increased when temperature was raised to 65 ± 0.5 °C. In case of MAE at low microwave power conditions, the extraction yield is function of extraction time (He and Xia, 2011).

Observed improvement in the extraction yield of total flavonoids with increased duration (from 10 min to 20 min) was in agreements with the fact that prolongation of MAE duration achieves better extraction yield at lower microwave power (He and Xia, 2011). Observed decline in the extraction yield at 105 ± 0.5 °C can be attributed to possible degradation of flavonoids at high temperature which may induce irradiation of high energy microwave (Biesaga, 2011, Pellati et al., 2013, Liazid et al., 2007). Further a high temperature of 105 ± 0.5 °C under pressure might have induced evaporation of some amount of ethanol which was observed as droplets condensed at the inner top of the lid. This might have reduced amount of solvent available during extraction thereby resulting in lower extraction yield.

Gelucire 44/14 based MAE of propolis:

Table 5.4. Flavonoids content (mg/g) for propolis extracts by Gelucire 44/14 based MAE.

Flavonoid compounds	Distilled Water (G0%w/w) (MW-DWP)	G 10% (w/w) solution (MW-GP10)	G 20% (w/w) solution (MW-GP20)	G 30% (w/w) solution (MW-GP30)	G 40% (w/w) solution (MW-GP40)	G 50% (w/w) solution (MW-GP50)
Caffeic acid	0.468 ± 0.015	0.512 ± 0.062	0.604 ± 0.027	0.829 ± 0.043	0.923 ± 0.082	0.643 ± 0.013
Quercetin	0.606 ± 0.011	0.619 ± 0.012	0.708 ± 0.115	0.736 ± 0.006	1.035 ± 0.047	0.912 ± 0.053
Apigenin	BDL	0.421 ± 0.011	0.500 ± 0.011	0.656 ± 0.04	1.293 ± 0.011	1.066 ±0.015
Kaempferol	0.523 ± 0.107	0.715 ± 0.026	0.931 ± 0.070	1.102 ± 0.061	3.878 ± 0.059	2.839 ± 0.23
Chrysin	BDL	2.071 ± 0.008	2.949 ± 0.045	4.471 ± 0.104	20.282 ± 0.353	14.296 ± 0.714
Acacetin	BDL	0.525 ± 0.054	0.627 ± 0.037	0.703 ± 0.006	1.811 ± 0.474	1.458 ± 0.409
Galangin	1.293 ± 0.025	3.792 ± 2.61	8.245 ± 0.048	11.698 ± 0.214	60.298 ± 1.694	41.308 ± 2.089
Cape	BDL	0.827 ± 0.084	1.032 ± 0.069	1.402 ± 0.134	5.332 ± 0.087	4.065 ± 0.074
Total flavonoids	2.891 ± 0.159	9.483 ± 2.425	15.597 ±0.157*	21.598 ±0.027**	94.854 ±1.589**	66.589 ±2.632**

Data expressed as Mean ±SD (n=3);
BDL: Below detection limit; Data was analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison test (*P< 0.05, ** P <0.001)

Table 5.4 depicts flavonoids content of MW-GP extracts obtained by Gelucire 44/14 based MAE. On account of poor solubility of flavonoids in water, the MW-DWP exhibited least extraction yields. Improvement in the extraction yield of total flavonoids was observed with introduction of Gelucire 44/14 in water. The Gelucire 44/14 solution of concentration 40% w/w achieved highest extraction of total flavonoids (94.854 ± 1.589 mg/g). Figure 5.6 displays chromatogram for blank Gelucire 44/14 solution demonstrating peak at Rt 54.86 ± 0.03 min, well resolved from that of any of the flavonoid marker of interest thus proving it to be a simple matrix suitable for analysis. Figure 5.7 depicts representative chromatogram for MW-GP40 extracted at respective wavelengths of detection for screened markers. Subsequent increase in amount of Gelucire 44/14 in water (50% w/w) resulted in reduced extraction yield for total flavonoids, though comparable to that of MW-EEP.

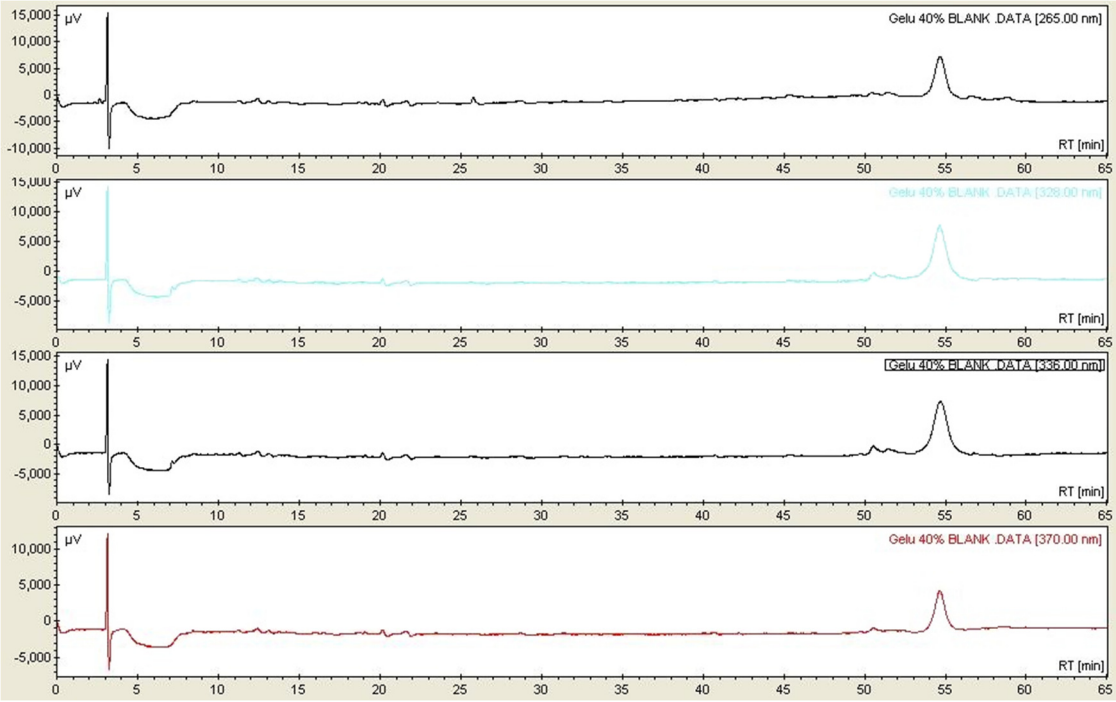


Figure 5.6. Stacked view of representative chromatogram for blank Gelucire 44/14 solution extracted at respective wavelengths of detection for screened markers

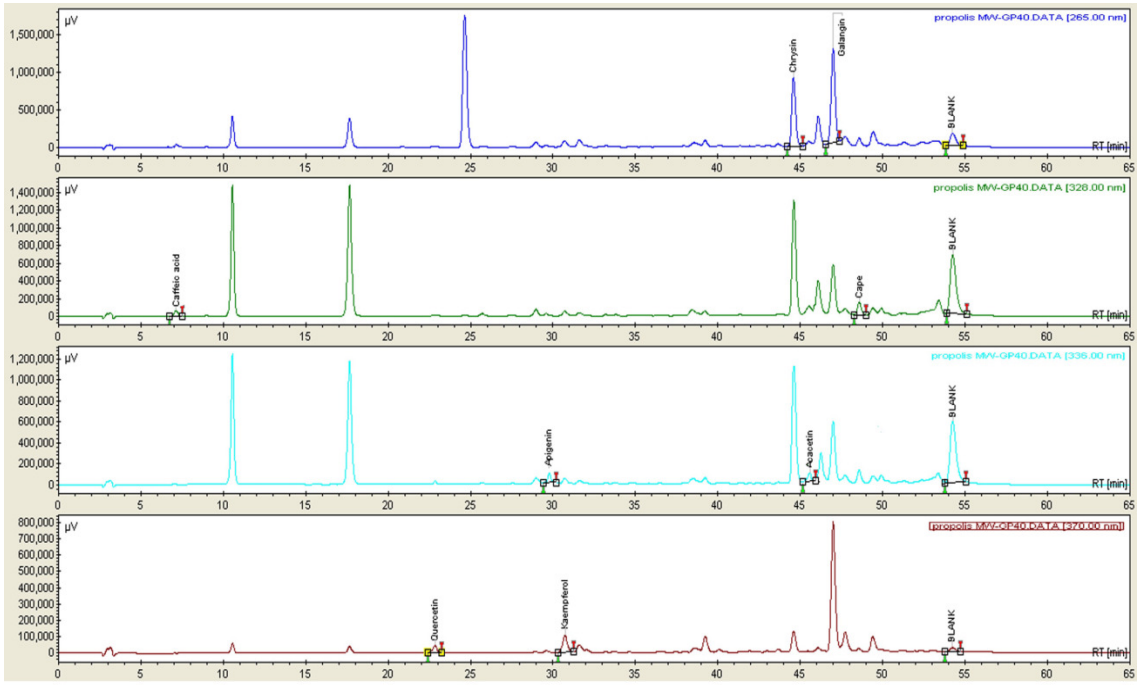
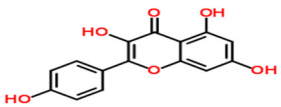


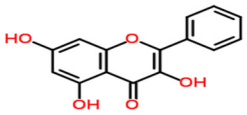
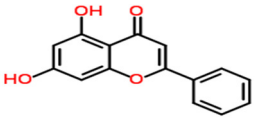
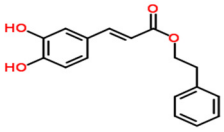
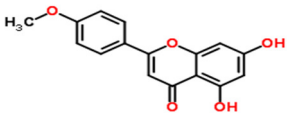
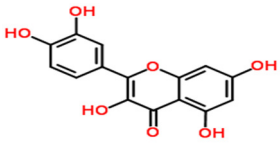
Figure 5.7. Stacked view of representative chromatogram for MW-GP40 extracted at respective wavelengths of detection for screened markers

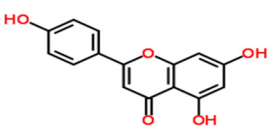
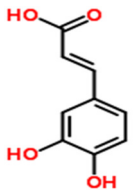
Chemically Gelucire 44/14 comprises of lauroyl polyoxyl-32-glycerides with HLB value of 14. On account of its ability to disperse or solubilize in an aqueous media by forming micelles, microscopic globules or vesicles, Gelucire 44/14 improves the solubility of poorly water-soluble constituents (Chambin and Jannin, 2005). Ampiphilic nature of Gelucire 44/14 offers an ability to engulf constituents like flavonoids with wide range of polarity and induce their micellar solubilization in aqueous media (Damian et al., 2000). Further on account of macrogol glyceride composition, Gelucire lipids possess prominent hydrogen bonding ability and acts as a proton donor (Shimpi et al., 2009). In contrast, the flavonoids contain several proton acceptor sites in their chemical structure on account of polyhydroxyl groups. The increased extraction yield of total flavonoids in Gelucire 44/14 solutions can be attributed to the possible intermolecular hydrogen bonding of flavonoids with Gelucire. Significant rise ($p<0.001$) in the total flavonoids content of MW-GP40 over MW-GP30 indicate enhanced intermolecular hydrogen bonding due to increased availability of proton donor groups with increasing amount of Gelucire 44/14. Further the number of

available proton acceptor sites in flavonoid structure and its log P value might have influenced its solubilization in the hydrophilic environment produced by Gelucire 44/14 (Shimpi et al., 2009). Table 6.5 demonstrates structure details and molecular descriptors for propolis flavonoids. Amongst the screened flavonoids kaempferol, chrysin, galangin, and cape have demonstrated around 3.5 to 5 folds increase in extraction yields of MW-GP40 over MW-GP30. This increase can be ascribed to the number of proton acceptor sites in their chemical structures (6 for kaempferol, 5 for galangin, 4 each for chrysin and cape) inducing stronger hydrogen bonding with Gelucire 44/14. Further the documented lower log P values for flavonoids (2.05 for kaempferol, 2.83 for galangin, 2.88 for chrysin and 3.38 for cape) suggests preferential solubilization in hydrophilic environment produced by Gelucire 44/14. Also there was observed around 1.1 to 2.5 fold increase in the extraction yield of caffeic acid, quercetin, apigenin and acacetin, possessing higher number of proton acceptor sites (4, 7, 5 and 5 respectively) and comparable log p values (1.42, 2.08, 2.10 and 3.15 respectively). Relatively low extraction yields of these compounds might be attributed to the possible low abundance or existence in the propolis sample. In light of the structure details for flavonoid molecules, increased extraction of flavonoids in Gelucire 44/14 solution can be ascribed to the intermolecular hydrogen bonding of flavonoids with Gelucire 44/14.

Table 5.5. Structure details and molecular descriptors for propolis flavonoids:

No	Flavonoid	Structure	Molecular weight	Log P value	'H' acceptor sites	'H' donor groups
1.	Kaempferol		286.24	2.05	6	4

2.	Galangin		270.237	2.83	5	3
3.	Chrysin		254.238	2.88	4	2
4.	Cape		284.306	3.38	4	2
5.	Acacetin		264.263	3.15	5	2
6.	Quercetin		302.24	2.08	7	5

7.	Apigenin		270.23	2.10	5	3
8.	Caffeic acid		180.16	1.42	4	3

(All the values for number of proton acceptor sites in chemical structure of flavonoids and their respective log P values are adopted from Royal Society of Chemistry's online chemical structure database http://www.chemspider.com/)

Decline in the total flavonoids content of MW-GP80 can be attributed to the increased viscosity of Gelucire 44/14 solution at concentration of 50% w/w, which might have retarded the mass transfer during extraction. Visually the MW-GP80 demonstrated thick and viscous consistency difficult to pour, hence the system was discarded. Comparative evaluation of tables 5.3 and 5.4 clearly demonstrates significant enhancement ($p< 0.001$) in the total flavonoids content of MW-GP40 when compared to that of MW-EEP65_20. This suggests an ability of Gelucire 44/14 as extractant to improve MAE of propolis constituents.

Dielectric properties of Gelucire 44/14 solutions and correlation with extraction of flavonoids

In order to understand the mechanism involved in Gelucire 44/14 based MAE, dielectric properties of the Gelucire 44/14 solutions were investigated. Dielectric properties or permittivity are the intrinsic properties of solvent which describe its behavior when subjected to electromagnetic field (Castro et al., 2011). They are expressed as relative complex permittivity (ε*):

$$(\varepsilon^*) = \varepsilon' - j\varepsilon'' \quad \ldots\ldots\ldots\ldots (1)$$

where ε' represents the dielectric constant; ε" reflects the dielectric loss factor and j represents function constant (√-1) (Guo et al., 2011; Kormin et al., 2013). Dielectric constant of solvent reflects its capacity to store the incident microwave energy while the dielectric loss denotes its ability to dissipate the microwave energy in the form of heat.

At a given microwave frequency, the temperature and solvent composition are the main parameters which influence dielectric properties of material or solvent (Icier and Baysal, 2004, Sosa et al., 2010, Venkatesh and Raghavan, 2004, Muley and Boldor, 2013). Therefore the dielectric properties of Gelucire 44/14 solutions were analyzed at temperature 25 ± 0.5 °C; 45 ±0.5 °C and 65 ± 0.5°C.

Table 5.6 depicts observed values of dielectric constant (ε') and loss factor (ε") for Gelucire 44/14 solutions in comparison with that of distilled water (i.e. without Gelucire 44/14).

Table 5.6: Dielectric properties for Gelucire 44/14 aqueous solutions

Temperature (± 0.5 °C)	Distilled Water (Gelucire 0% w/w)		Gelucire 10% (w/w)		Gelucire 20% (w/w)		Gelucire 30% (w/w)		Gelucire 40% (w/w)		Gelucire 50% (w/w)	
	Dielectric constant (ε')	Dielectric Loss (ε'')	Dielectric constant (ε')	Dielectric Loss (ε'')	Dielectric constant (ε')	Dielectric Loss (ε'')	Dielectric constant (ε')	Dielectric Loss (ε'')	Dielectric constant (ε')	Dielectric Loss (ε'')	Dielectric constant (ε')	Dielectric Loss (ε'')
25	76.93 ± 0.27	7.72 ± 0.26	65.12 ± 0.30	6.90 ± 0.13	60.86 ± 0.07	7.05 ± 0.13	54.46 ± 0.17	8.03 ± 0.09	38.6 ± 0.39	9.14 ± 0.16	13.68 ± 0.11	5.28 ± 0.13
45	72.28 ± 0.32	4.56 ± 0.6	58.55 ± 0.49	6.35 ± 0.13	53.72 ± 0.72	5.47 ± 0.15	48.47 ± 0.23	6.27 ± 0.14	34.61 ± 0.33	7.44 ± 0.42	12.72± 0.26	4.77 ± 0.12
65	66.69 ± 0.43	3.05 ± 0.61	54.62 ± 0.44	3.98 ± 0.17	51.62 ± 0.37	4.21 ± 0.58	50.13 ± 0.52	4.70 ± 0.20	32.8 ± 0.13	6.76 ± 0.29	11.51 ± 0.11	4.24 ± 0.10

Data expressed as Mean ± SD (n=3)

From the table 5.6, it was observed that at a given temperature, the concentration of Gelucire 44/14 has profound effect on (ε') and (ε"), whereas variation in temperature did not show similar effect at a given concentration. The results display water being most polar solvent possesses highest dielectric constant of 66.69 ± 0.43 at 65 ± 0.5°C. Incorporation of Gelucire 44/14 reduced the dielectric constant of water gradually. The high dielectric constant of water can be ascribed to the tetrahedral hydrogen bonded structure in liquid state. A water molecule comprises two lone pairs of electron and two donatable hydrogen atoms which enables formation of four hydrogen bonds in liquid form. On account of this ability, the water molecule demonstrates cooperative hydrogen bonding (Franks and Ives, 1966). The cooperative hydrogen bonding phenomenon imparts tetrahedral hydrogen bonded structure to water molecule exhibiting high dielectric constant. Disturbance of this tetrahedral structure by addition of solute or solvent alters the dielectric constant of water (Malmberg and Maryott, 1950). Observed reduction in ε' of water by addition of Gelucire 44/14 suggests interruption of tetrahedral structure of water molecules reducing its polarity. Further, Gelucire 44/14 exhibits a polyhydric composition comprising mixture of polyethylene glycol (PEG 33), PEG mono and di-esters of fatty acids, glycerides and small proportion of glycerol (Chambin and Jannin, 2005). Therefore increments of Gelucire 44/14 would increase the amount of glyceride chains in water thereby reducing ionic conduction and dipole rotation of free water molecules. Similar reduction in ε' of solvent with addition of ethylene glycol oligomers was reported at constant frequency and temperature (Sengwa and Kaur, 1999).

Correlating dielectric properties of Gelucire 44/14 solutions with the flavonoids extraction yield:

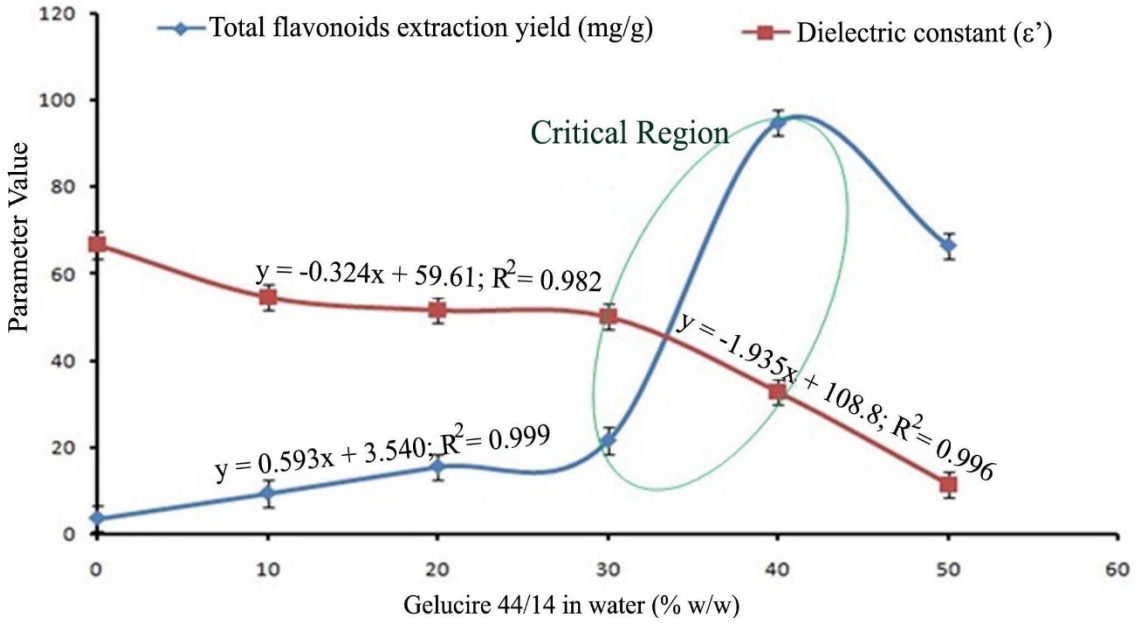


Figure 5.8A. Correlation between dielectric constant and flavonoids extraction yield as a function of Gelucire 44/14 concentration in water at 65 ± 0.5°C

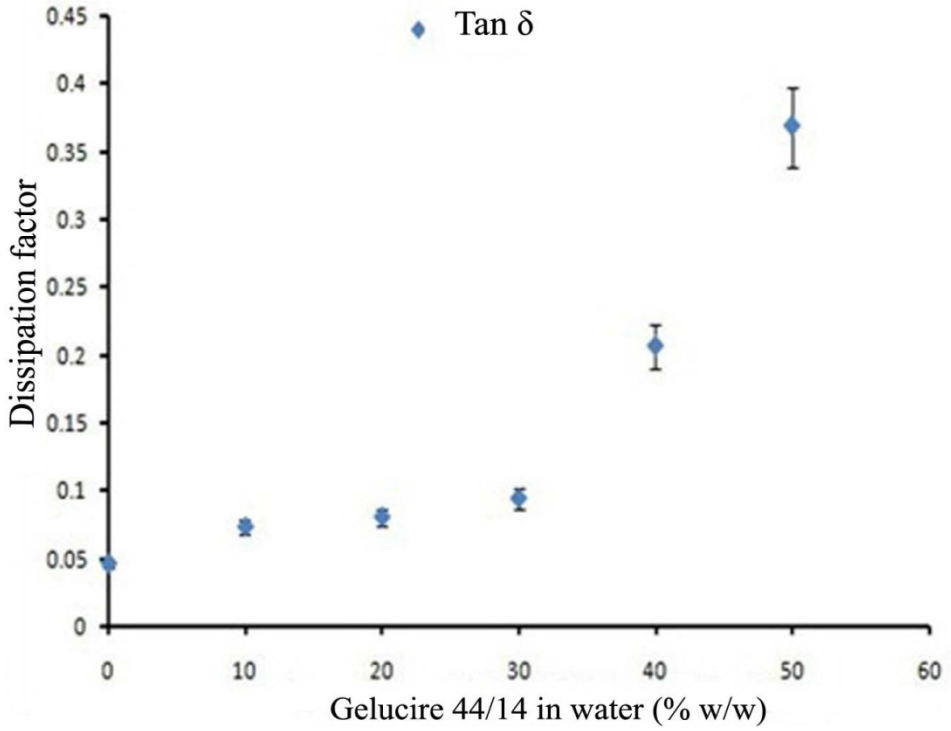


Figure 5.8B. Modulation of dissipation factor as a function of Gelucire 44/14 concentration in water at 65 ± 0.5°C

Table 5.7. Equations defining the modulation of dielectric constant (ε') and flavonoids extraction yields as a function of Gelucire 44/14 concentration:

Gelucire 44/14 (% w/w)	Linear regression for reduction of (ε')	R^2	Linear regression for increase in flavonoids extraction	R^2
10 to 30	y = -0.324x + 59.61	0.982	y = 0.593 + 3.540	0.999
30 to 50	y = -1.935x + 108.8	0.996	--	--

Figure 5.8A demonstrates correlation between ε' of Gelucire 44/14 solutions with the extraction yield of flavonoids plotted as a function of Gelucire 44/14 concentration in water at 65 ± 0.5°C. The curve for ε' demonstrates bimodal behavior with a point of inflection at concentration of 30% w/w of Gelucire 44/14 in water. Linear regression analysis as displayed in table 5.7, showed that addition of 10 to 30% w/w Gelucire 44/14 reduced ε' with a factor of 0.324. Further increments of Gelucire 44/14 above 30% w/w to 50% w/w induced around 6 folds drastic reduction in ε' with a factor of 1.935. Similarly curve for total flavonoids extraction yield showed that increments of Gelucire 44/14 in the concentration range of 10 to 30% w/w linearly increased the flavonoids extraction with a factor of 0.593. However significant increase ($p < 0.001$) in total flavonoids content was observed at Gelucire 44/14 concentration of 40% w/w. Further increase in Gelucire 44/14 concentration lead to reduced extraction of flavonoids as discussed previously. Figure 5.8A depicts critical concentration range for Gelucire 44/14 solutions (30 to 40% w/w) wherein significant changes in ε' and flavonoids extraction yield was observed. Rapid reduction in ε' above 30% w/w of Gelucire 44/14 suggests enhanced interaction of its hydrophilic groups with the water molecules leading to effective interruption of tetrahedral structure (as verified by from DSC studies). This in turn reduces the polarity of water to a degree favorable for facilitating solubilization of flavonoids thereby increasing the extraction yield at 40% w/w of Gelucire 44/14.

The dielectric loss indicates amount of microwave energy dissipated as heat to the sample (Mandal et al., 2007; Kormin et al., 2013). The efficiency of microwave heating

depends upon dissipation factor i.e. 'tangent of loss angle' (Tan δ) which denotes relation between ε' and ε" as:

$$\text{Tan } \delta = \varepsilon''/\varepsilon' \quad \ldots\ldots\ldots\ldots (2)$$ (Mandal et al., 2007, Chan et al., 2011)

Thus both ε" and tan δ express amount of heat generated when the solvent is exposed to microwave (Kormin et al., 2013). For dielectric materials with comparable loss factor values (as found in table 5.6), Tan δ serves as convenient parameter indicating the efficient conversion of microwave energy into thermal energy (Grant and Halstead, 1998).

Figure 5.8B correlates concentration of Gelucire 44/14 solution with the calculated values for Tan δ at 65 ± 0.5 °C. The curve clearly shows that Tan δ of solution increases with increase in amount of Gelucire 44/14 in water. Loss tangent value of solvent depends upon the relaxation time of molecules which ultimately rely on the type of functional groups and volume of the molecules. Functional groups favoring hydrogen bonding exhibit strong influence on relaxation times (Grant and Halstead, 1998). Thus, evident rise in tan δ of water with increments of Gelucire 44/14 can be attributed to increase in bulky lauroylpolyoxyl glyceride groups. Rising Tan δ values indicate improved conversion of absorbed microwave energy into thermal energy upon addition of Gelucire 44/14. The increased heat generation contributes to elevate the temperature of solution to 65 ± 0.5 °C where maximum extraction of flavonoids was observed.

Differential scanning calorimetry:

In order to understand the micro-structural interaction of Gelucire 44/14 with water molecules we performed subzero temperature DSC analysis as per reported method (Patil et al., 2012a, Patil et al., 2012b). Position of endothermic peaks in DSC thermogram can describe association of water molecules with surfactant chains (Patil et al., 2012b). An endotherm below -10 °C represents water bound with hydrophilic groups of surfactant whereas the free water melts at 0 °C (Senatra et al., 1991, Patil et al., 2012a, Patil et al., 2012b). Figure 5.9 represents DSC thermogram for the Gelucire 44/14 solutions (10 to 50 % w/w).

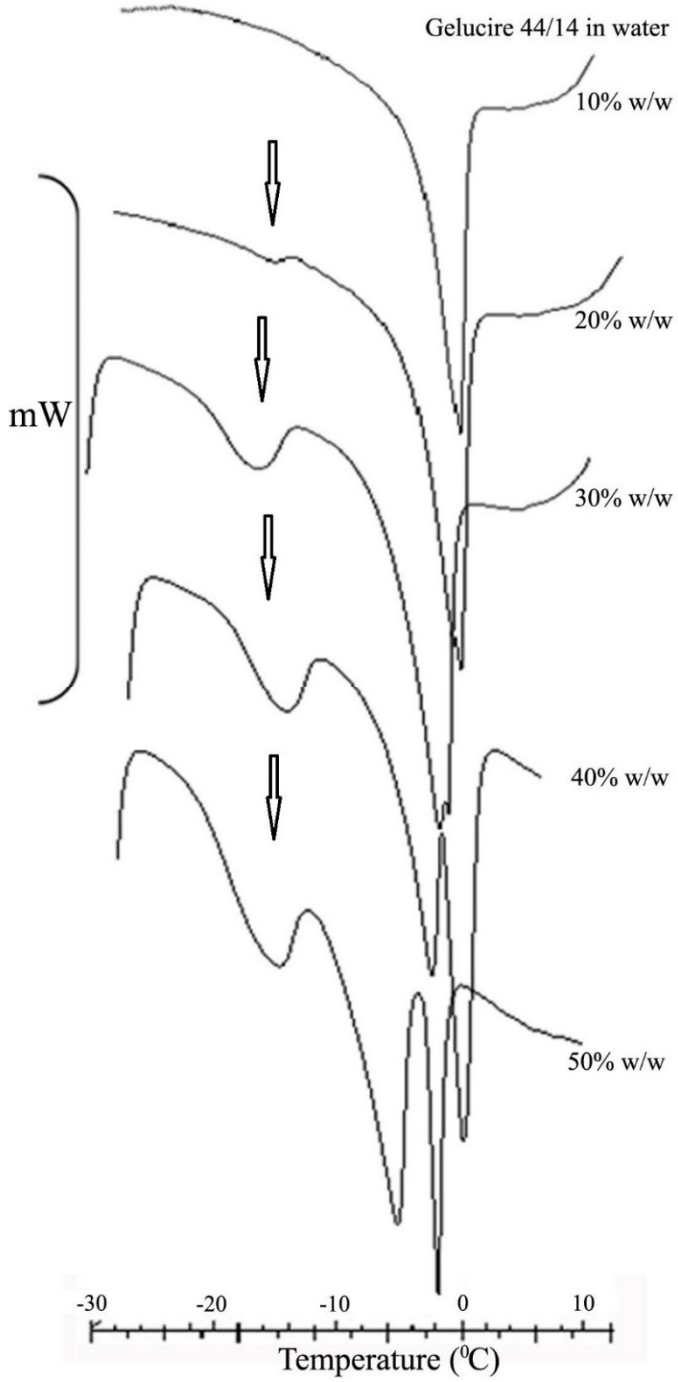


Figure 5.9. DSC thermogram for Gelucire 44/14 solutions (10 to 50 % w/w).

As observed in figure 5.9, all the endothermic peaks appearing below -10 °C (marked with arrow) indicate bound water i.e. closely linked with surfactant chains of Gelucire44/14. These endothermic events of the Gelucire 44/14 solutions are summarized in Table 5.8.

Table5.8. Endothermic event of Gelucire 44/14 aqueous solutions at subzero temperature

No	Concentration of Gelucire 44/14 in water (w/w)	Endothermic peak for bound water at temperature (°C)	Enthalpy (Jg^{-1})
1	10%	--	--
2	20%	-15.78	-3.51
3	30%	-17.04	-14.82
4	40%	-16.68	-26.84
5	50%	-16.79	-24.22

Enthalpy value for each solution reflects the degree of binding strength of water with surfactant chains at respective concentration (Patil et al., 2012a). There was observed significant rise in the enthalpy (-26.84 Jg^{-1}) of solution with addition of Gelucire 44/14 at a concentration of 40% w/w. This indicates stronger association of water molecules with the surfactant chains at 40% w/w. This finding corroborate with observed low value of ε' at 40% w/w of Gelucire 44/14 suggesting effective interruption of tetrahedral structure of water molecules. Similar breaking of tetrahedral network structure of water with addition of glycerol has been reported (Elamin et al., 2013).

To summarize, the dielectric properties of solvent in terms of dielectric constant, loss and dissipation factor determine its performance in MAE (Singh et al., 2014). From the findings of current study it can be postulated that Gelucire 44/14 as an extractant in MAE demonstrates multi-functionality:

a) Modulates dielectric constant of water by disrupting the tetraherdral structure which ultimately reduces its polarity to facilitate solubilization of flavonoids in solution.

b) Achieves effective conversion of absorbed microwave energy into thermal energy enhancing the extraction efficiency.

c) Induces intermolecular hydrogen bonding with flavonoids on account of its proton donating ability which increases their extraction yields.

d) Being a non-ionic surfactant Gelucire 44/14 might also facilitate initial penetration of water into the propolis matrix by reducing the surface tension which induces wetting of constituents present within. This augments swelling of the matrix and intensifies the process of mass transfer resulting in improved extraction of actives (Ajila et al., 2011, Ketkar et al., 2011).

It is documented that apart from composition of solvent, addition of plant matrix may also contribute to alter the dielectric properties of solvent at defined set of conditions (Singh et al., 2014). The plant matrix also absorbs microwave energy, which is later dissipated as heat. Introduction of potato peels was reported to slow down the reduction in ε' of methanol-water mixture while recorded higher values for ε" and tan δ than those obtained without potato peels at 2.45 GHz (Singh et al., 2014). Thus addition of propolis to Gelucire 44/14 solution might contribute to alter its dielectric properties thereby achieving rapid heating by microwaves. This might have induced disruption of cellular matrix and leaching of flavonoids into the medium.

Thus the Gelucire 44/14 based MAE of propolisproposed herein coalesce the benefits of "MAE" with merits of organic solvent free technique. Peculiar characteristics of the method involves:

a) an aqueous solvent system in MAE extracting most water insoluble flavonoids from propolis;

b) achieves higher extraction yields;

c) avoids use of organic solvent making MAE greener technique;

d) the propolis extract produced (MW-GP) hereby possess no risk of residual toxic organic solvents;

e) the extract is easily dispersible in aqueous fluids, envisaging better biopharmaceutical performance ascribed to Gelucire 44/14, however detailed investigations about same need to be performed.

5B.5. Conclusion:

The study provides herein a green extraction method for propolis. It recognizes Gelucire 44/14 as promising extractant in MAE of flavonoids from propolis. Compared with the conventional MAE procedure, proposed Gelucire 44/14 based MAE of propolis provided higher extraction yields without use of organic solvents. Importantly, investigation of dielectric properties of Gelucire 44/14 solutions revealed their microwave absorptive and dissipation abilities. Incorporation of Gelucire 44/14 in water at 40% w/w was found to modulate its dielectric properties to a degree favorable for increasing the extraction of flavonoids. The subzero DSC analysis revealed phenomenon of dielectric constant reduction by Gelucire 44/14 suggesting interruption of tetrahedral structure of water molecules reducing its polarity which in turn facilitates solubilization and extraction of flavonoids.

References

Ajila C, Brar S, Verma M, Tyagi R and Valero J (2011). Solid-state fermentation of apple pomace using Phanerocheate chrysosporium–Liberation and extraction of phenolic antioxidants. *Food Chemistry,* 126**:**1071-80.

Biesaga M (2011). Influence of extraction methods on stability of flavonoids. *Journal of Chromatography A,* 1218**:**2505-12.

Castro GM, Dols L, Toldra F and Fito P (2011). Development of a dielectric spectroscopy technique for the determination of key biochemical markers of meat quality. *Food Chemistry,* 127**:**228-33.

Chambin O and Jannin V (2005). Interest of multifunctional lipid excipients: case of Gelucire® 44/14. *Drug development and industrial pharmacy,* 31**:**527-34.

Chan CH, Yusoff R, Ngoh GC and Kung FWL (2011). Microwave-assisted extractions of active ingredients from plants. *Journal of Chromatography A,* 1218**:**6213-25.

Damian F, Blaton N, Naesens L, Balzarini J, Kinget R, Augustijns P and Van Den Mooter G (2000). Physicochemical characterization of solid dispersions of the antiviral agent UC-781 with polyethylene glycol 6000 and Gelucire 44/14. *European journal of pharmaceutical sciences,* 10**:**311-22.

Datta AK (2001). Handbook of microwave technology for food application. CRC Press.

Elamin K, Jansson H, Kittaka S and Swenson J (2013). Different behavior of water in confined solutions of high and low solute concentrations. *Physical Chemistry Chemical Physics,* 15**:**18437-44.

Franks FT and Ives D (1966). The structural properties of alcohol–water mixtures. *Quarterly Reviews, Chemical Society,* 20**:**1-44.

Gardana C, Simonetti P, Berti C and Pietta P (2007). Evaluation of propolis polyphenols absorption in humans by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry,* 21**:**3849-54.

Grant E and Halstead BJ (1998). Dielectric parameters relevant to microwave dielectric heating. *Chemical Society Reviews,* 27**:**213-24.

Guidelines ICH 1996. Q2 (R1)(2005) Validation of analytical procedures: text and methodology. *International conference on harmonization.*

Guo W, Liu Y, Zhu X and Wang S (2011). Dielectric properties of honey adulterated with sucrose syrup. *Journal of Food Engineering,* 107**:**1-7.

He Z and Xia W (2011). Microwave-assisted extraction of phenolics from Canarium album L. and identification of the main phenolic compound. *Natural product research,* 25**:**85-92.

Icier F and Baysal T (2004). Dielectrical properties of food materials—1: Factors affecting and industrial uses. *Critical reviews in food science and nutrition,* 44**:**465-71.

Ketkar S, Gopu C, Badgujar L, Paradkar A and Mahadik K (2011). Male sexual behavior improving effect of lipid based extract of Mucuna Pruriens in rats. *Pharmacologyonline,* 1**:**1-14.

Kormin, F., Abdurahman, NH., Yunus, RM., Rivai, M. Study the heating mechanisms of temperature controlled microwave closed system (TCMCS). International Journal of Engineering Science and Innovative Technology (IJESIT). 2013; 2(5)

Liazid A, Palma M, Brigui J and Barroso CG (2007). Investigation on phenolic compounds stability during microwave-assisted extraction. *Journal of Chromatography A,* 1140**:**29-34.

Malmberg CG and Maryott AA (1950). Dielectric constants of aqueous solutions of dextrose and sucrose. *Journal of Research of the National Bureau of Standards,* 45**:**299-303.

Mandal V, Mohan Y and Hemalatha S (2007). Microwave assisted extraction—an innovative and promising extraction tool for medicinal plant research. *Pharmacognosy Reviews,* 1**:**7-18.

Muley PD and Boldor D (2013). Investigation of microwave dielectric properties of biodiesel components. *Bioresource technology,* 127**:**165-74.

Patil SS, Venugopal E, Bhat S, Mahadik KR and Paradkar AR (2012a). Microstructural elucidation of self-emulsifying system: effect of chemical structure. *Pharmaceutical research,* 29**:**2180-88.

Patil SS, Venugopal E, Bhat S, Mahadik KR and Paradkar AR (2012b). Probing influence of mesophasic transformation on performance of self-emulsifying system: effect of ion. *Molecular pharmaceutics,* 9**:**318-24.

Pellati F, Prencipe FP, Bertelli D and Benvenuti S (2013). An efficient chemical analysis of phenolic acids and flavonoids in raw propolis by microwave-assisted extraction combined with high-performance liquid chromatography using the fused-core technology. *Journal of pharmaceutical and biomedical analysis,* 81**:**126-32.

Senatra D, Lendinara L and Giri M (1991). W/O microemulsions as model systems for the study of water confined in microenvironments: Low resolution 1H magnetic resonance relaxation analysis. Trends in Colloid and Interface Science V. Springer, 122-28.

Sengwa R and Kaur K (1999). Microwave absorption in oligomers of ethylene glycol. *Indian Journal Of Biochemistry And BiophysicS,* 36**:**325-29.

Shimpi SL, Mahadik KR and Paradkar AR (2009). Study on mechanism for amorphous drug stabilization using Gelucire 50/13. *Chemical and Pharmaceutical Bulletin,* 57**:**937-42.

Singh A, Nair GR, Liplap P, Gariepy Y, Orsat V and Raghavan V (2014). Effect of Dielectric Properties of a Solvent-Water Mixture Used in Microwave-Assisted Extraction of Antioxidants from Potato Peels. *Antioxidants,* 3**:**99-113.

Sosa MM, Valerio JL, Lopez MA and Garcia H (2010). Dielectric properties of foods: reported data in the 21st century and their potential applications. *LWT-Food Science and Technology,* 43**:**1169-79.

Trusheva B, Trunkova D and Bankova V (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry central journal,* 1**:**13.

Venkatesh M and Raghavan G (2004). An overview of microwave processing and dielectric properties of agri-food materials. *Biosystems Engineering,* 88**:**1-18.

Chapter 6. Caffeic acid phenethyl ester (CAPE): Development of cocrystals

This chapter will cover studies performed on CAPE. The chapter discusses application of microwave assisted cocrystallization technique for generation of CAPE cocrystals. It includes development of cocrystals for CAPE using pharmaceutically acceptable coformers. Screening of coformers, synthesis of cocrystals and their characterization by spectroscopic and diffraction techniques has been discussed. This is followed by determination of aqueous solubility of the cocrystals in comparison with the parent compound.

6.1. Experimental work

6.1.1. Synthesis of CAPE Cocrystals:

Different co-formers (Table 6.1) were screened for generation of co-crystals with CAPE by microwave irradiation technique as per reported method (Pagire et al., 2013).

Procedure:

i. Individually, equimolar quantities (0.1 mmol each) of CAPE and respective co-formers (as listed in table 6.1) were mixed in separate glass tubes of capacity 30ml.

ii. To the individual mixtures, 60 % (w/v) of solvent was added and subjected to microwave irradiation in microwave reactor (Monowave 300, Anton Paar, Gmbh, Austria) to induce cocrystallization. The operating conditions viz. target temperature (80 °C), hold time (60 sec) followed by cooling to 40°C were adopted.

iii. Upon completion of the microwave irradiation period, the lid was opened and the resulting mass was collected and subjected to the characterization techniques.

Figure 6.1. Monowave 300, Anton Paar, Gmbh, Austria.

Table 6.1: Coformers screened for cocrystallization with CAPE (1:1)

No.	Coformer	Melting point (°C)	Solvent (60% w/v)
1	Caffeine	235-238	Ethanol
2	Nicotinamide	130-131	Ethanol
3.	Isonicotinamide	155-157	Ethanol

4.	Gallic acid	257-258	Ethanol
			Methanol
			Acetone
			Ethyl acetate
5.	p-Coumaric acid	210-212	Ethanol
			Methanol
			Acetone
			Ethyl acetate
6.	Curcumin	182-184	Ethanol
			Methanol
			Ethyl acetate
7.	Isoniazid	171-172	Ethanol
8.	Theophylline	273-274	Ethanol
9.	Urea	132-134	Ethanol
10.	Ibuprofen	75-76	Ethanol

6.1.2. Characterization:

6.1.2.1. Powder X-ray diffraction (PXRD):

The microwave processed materials were screened for cocrystal formation by employing Bruker D8 advanced diffractometer, X-ray wavelength (λ) of 0.154nm, source: Cu, voltage: 40kV with filament emission of 40mA. The samples were scanned from 2 to 30° 2θ at the scanning speed of 0.01° step width.

6.1.2.2. Differential scanning Calorimetry (DSC):

The microwave processed materials were subjected to thermal analysis using TA Instruments DSC 2000 differential scanning calorimeter. The samples were subjected to heating from 20 °C to their respective melting temperature at rate of 10 °C/ min in DSC cell under inert nitrogen environment. Aluminum pans were used for all samples.

6.1.2.3. Raman spectroscopic characterization:

Raman spectra for the CAPE and its cocrystals along with the screened coformers were recorded using a Raman microscope and Renishaw InVia Reflex bench top spectrometer coupled with 683 nm stabilized diode (Renishaw Plc., UK). A laser spot of

diameter (footprint) 2 μm was obtained at specimen using a 100X objective lens. The spectra were collected using extended scanning mode over the wavenumber region of 3500–100 cm^{-1}. 10 scans were collected for each sample. Data analysis was performed using Galactic Grams AI 8.0 spectroscopy software (Thermo Electron Corporation).

6.1.3. Equilibrium Solubility measurements:

All the cocrystals were synthesized in bulk using stoichiometric ratios of CAPE and respective coformers in ethanol by subjecting to microwave irradiation as described in section 6.1.1. CAPE and the cocrystal samples were individually added in small portions to 1 ml distilled water at room temperature (25 ± 0.5°C) and stirred at 600 rpm. The addition of solids was stopped once they showed precipitation in the solvent. Individually the systems were stirred for further 24 hrs to ensure the equilibration of solids with solvent. The samples were filtered using 0.45 μ nylon filter. The filtrate was suitably diluted and subjected to HPLC analysis on Waters e-2695 system integrated with a PDA 2998 detector, Empower 3 software and Waters symmetry C18 column (4.6 × 250 mm, 5 μm) maintained at 25 ± 0.5C° throughout study. Elution was carried out using mobile phase methanol: acetonitrile (50:50 v/v) in an isocratic method at a flow rate of 1 ml/min (Ceschel et al., 2002).The detection was carried out at 246 nm. The experiment was performed in triplicates. Calibration curve was determined for the CAPE by plotting known concentration of each dilution vs. corresponding peak area.

6.1.4. X-Ray Crystallography

Single crystal X-ray data on CAPE-NIC cocrystal was collected on the Xcalibur Gemini EOS CCD diffractometer (Oxford Diffraction, Yarnton, UK) using Mo-Kα, radiation at 298K. Data reduction was performed using CrysAlisPro (version 1.171.33.55). OLEX2-1.0, and SHELX-TL 97 were used to solve and refine the reflections data. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms on O and N were experimentally located in difference electron density maps. All C–H atoms were fixed geometrically using HFIX command in SHELX-TL. A check of the final CIF file using

PLATON did not show any missed symmetry. X-Seed was used to prepare packing diagrams.

6.2. Results and discussion:

Pharmaceutical co-crystals comprise an active pharmaceutical ingredient (API) and coformer held together in definite stoichiometry, in same crystal lattice through intermolecular interactions (Almarsson and Zaworotko, 2004, Jones et al., 2006, Shan and Zaworotko, 2008). Recent US FDA guidance document (2013) on cocrystals define cocrystal as dissociable API-excipient molecular complex. Co-crystals exhibit non-covalent interactions like hydrogen bonding, halogen bonding and π-π interactions between the active ingredient and co-former (Trask and Jones, 2005, Vishweshwar et al., 2006). Cocrystalization of an API with different coformers has been found an effective strategy for modulating physicochemical properties of API thereby improving its biopharmaceutical and therapeutic performance (Schultheiss and Newman, 2009). Several reports dictate improvement in solubility and dissolution rate of API through cocrystallizaton with suitable coformers. (Blagden et al., 2007, Good DJ and Rodriguez HN 2009, Sanphui et al., 2011). Cocrystalization of theophylline was reported to improve its physical stability by reducing its succeseptibility to hydration. (Trask et al., 2006). Development of quercetin cocrystals were reported to improve its oral bioavailability by around 10 folds as compared with that of the native quercetin (Smith et al., 2011). Similiarly, cocrystallization of epigallocatechin-3-gallate with suitable coformers improved its bioavailability (Smith et al., 2011, Smith et al., 2013). Further cocrystallization of paracetamol with oxalic acid was reported to improve its compressibility. (Karki et al., 2009). Considering these evidences, attempts were made in the present study to develop cocrystals of CAPE using suitable coformers with an aim to improve its aqueous solubility.

Microwaves comprise electromagnetic radiations with frequency ranging between 0.3 to 300 GHz. Typically microwaves of frequency 2.45 GHz are preferred in household and industrial applications (Datta, 2001, Kappe, 2004). Recently, microwave

irradiation has been proved to be a rapid and less solvent consuming method of cocrystallization (Pagire et al., 2013). Microwave irradiation of active ingredient with coformer initiates two simultaneous processes viz, a) attainment of supersaturation levels of the reacting components and formed cocrystal phase; b) evaporation of solvent contributing to supersaturation of the co-crystal phase. Thus the microwave induced dielectric heating results in rapid saturation of interacting components and maintain the levels throughout the process. Therefore in the current work efforts were made to produce CAPE cocrystals by microwave assisted cocrysyallilzation technique.

Screening of different coformers:

Selection of coformer is a critical step in development of co-crystal. During selection of coformers, primarily the potential sites for hydrogen bonding from the CAPE were identified. It was followed by selection of coformers possessing complementary functional groups capable of forming hydrogen bonds with CAPE. Considering the nature of functional groups in CAPE molecule, coformers with complementary functional moieties were selected so as to form cocrystals. The primary functional groups in CAPE are 1,2-benzenediol and ester moieties. In literature, COOH, $CONH_2$ and pyridine functional moieties have been reported to assemble with hydroxyl containing molecules (Etter, 1991, Goud et al., 2012). Accordingly, CAPE was cocrystallized with suitable coformers containing these functional groups. Table 6.2 provides experimental observations and inferences against different coformers screened with CAPE for cocrystallization.

Table 6.2: Experimental observations for screening of coformers for cocrystallization:

No.	Coformer	Solvent (60% w/v)	Observations	Inference
1	Caffeine	Ethanol	i) DSC thermogram showed melting endotherm at 113.48 °C. ii) PXRD showed distinct peak at 2θ value	Formation of CAPE:CAF cocrystal was evident (however

			3.5.	diffraction quality single crystal was not produced)
2	Nicotinamide	Ethanol	i) DSC thermogram showed melting endotherm at 106.67 °C. ii) PXRD showed distinct peak at 2θ value 4.6.	Formation of CAPE:NIC cocrystal was evident Also diffraction quality single crystal was produced.
3.	Isonicotinamide	Ethanol	i) DSC thermogram showed melting endotherm at 107.98 °C. ii) PXRD showed distinct peak at 2θ value 4.6.	Formation of CAPE:CAF cocrystal was evident. (however diffraction quality single crystal was not produced)
4.	Gallic acid	Ethanol	DSC thermogram showed endotherm peaks at 113.8, 117.14 and 275.6 °C.	No cocrystal was produced.
		Methanol	DSC thermogram showed sharp endotherm peaks at 127.88 °C and degradation above 225 °C	No cocrystal was produced.
		Acetone	DSC thermogram showed sharp endotherm peak 128.23°C	No cocrystal was produced.
		Ethyl acetate	DSC thermogram showed sharp endotherm peak 128.23°C	No cocrystal was produced.
5.	p-Coumaric acid	Ethanol	DSC thermogram showed endotherm peak at 126.84 °C and degradation above 190 °C.	No cocrystal was produced.
	p-Coumaric acid	Methanol	DSC thermogram showed endotherm peak at 125.45 °C and	No cocrystal was produced.

			degradation above 190 °C.	
	p-Coumaric acid	Acetone	DSC thermogram showed endotherm peak at 126.50 °C and degradation above 194 °C.	No cocrystal was produced.
	p-Coumaric acid	Ethyl acetate	DSC thermogram showed endotherm peak at 125.80 °C and degradation above 195 °C.	No cocrystal was produced.
6.	Curcumin	Ethanol	DSC thermogram showed endotherm peak at 122.47 °C.	No cocrystal was produced.
	Curcumin	Methanol	DSC thermogram showed endotherm peak at 122.47 °C.	No cocrystal was produced.
	Curcumin	Ethyl acetate	DSC thermogram showed endotherm peak at 122.47 °C.	No cocrystal was produced.
7.	Isoniazid	Ethanol	DSC thermogram showed endotherm peak at 101.47 °C.	No cocrystal was produced.
8.	Theophylline	Ethanol	DSC thermogram showed endotherm peak at 117.30 °C.	No cocrystal was produced.
9.	Urea	Ethanol	DSC thermogram showed endotherm peak at 99.95 and 133.14 °C.	No cocrystal was produced.
10.	Ibuprofen	Ethanol	DSC thermogram showed endotherm peak at 74 and 121.69 °C.	No cocrystal was produced.

Differential Scanning Calorimetry:

Differential scanning calorimetric analysis displayed unique thermal behavior and the phase purity of CAPE cocrystals. Table 6.3 describes melting points of CAPE cocrystals in comparison with that of respective conformer.

Table 6.3: Melting points of CAPE cocrystals:

S. No	CAPE cocrystal	Melting Point (°C)	Coformer	Melting Point (°C)
1	CAPE-NIC	106.67	Nicotinamide	130.54
2	CAPE-INIC	107.98	Isonicotinamide	155-157
3	CAPE-CAF	113.48	Caffeine	235-238

Melting point of CAPE is 129°C

Interestingly, it was observed that none of the cocrystals exhibited a melting point in between that of the corresponding individual components as observed in certain cocrystal systems (Walsh et al., 2003). Instead, all the cocrystal phases exhibited lower melting point as compared to the parent CAPE and the respective coformer. The cocrystal melting points showed a direct dependence on the coformer melting point, i.e. higher melting cocrystal was formed by a higher melting conformer. Figure 6.2 demonstrates DSC thermogram for CAPE, its cocrystals along with the individual coformers. The DSC thermogram clearly depicted the phase purity of CAPE cocrystals.

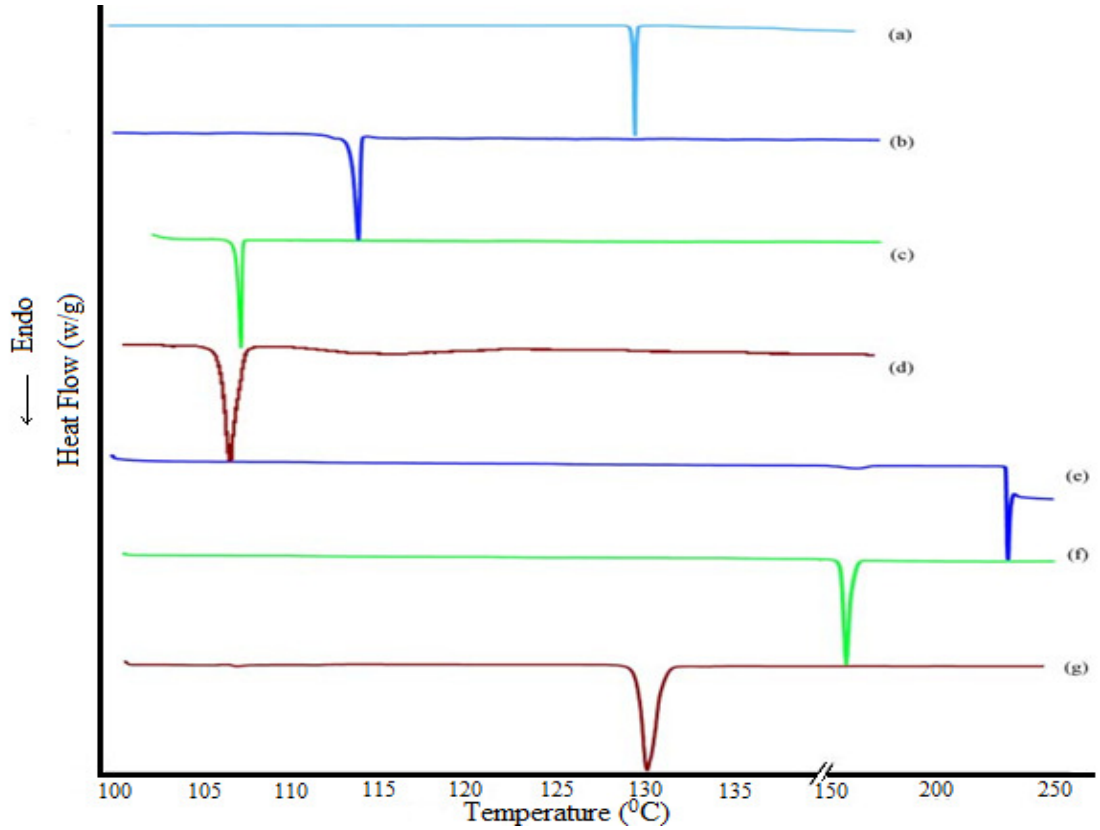


Figure 6.2. DSC Heating curves for a) pure CAPE; b) CAPE-CAF; c) CAPE-INIC; d) CAPE-NIC; e) CAF f) INIC and g) NIC

Powder X-ray Diffraction:

Powder diffraction is a reliable technique to ascertain the novelty of cocrystals through their unique diffraction patterns in comparison to their starting materials. It is the method of choice for those cases where single crystals are not available by crystallization. In addition to cocrystals, it also finds application in analyzing bulk purity of polymorphs, amorphous content in a crystalline material and structure of a crystalline material from its diffractograms (Norby, 2006, Karki et al., 2007). PXRD was employed as a fingerprint tool to examine the crystallinity and novelty of CAPE cocrystals.

The experimental powder patterns of bulk materials of CAPE cocrystals and their respective individual components are shown in Figure 6.3. The PXRD pattern of CAPE exhibited characteristic reflections at about 2θ 6.1°, 18.38°, 18.44°, 21.7° and 26.64 (Figure 6.3a). The PXRD pattern for CAPE-CAF exhibited characteristic reflections at different 2θ values viz.3.6°, 16.9°, 22.1° and 26.5° (Figure 6.3b). Also PXRD pattern for CAPE-INIC exhibited characteristic reflections at about 2θ 4.15°, 16.35°, 24° and 26.4° (Figure 6.3c). Further the CAPE-NIC demonstrated different PXRD pattern with characteristic reflections at 2θ 4.1°, 16.4°, 19.9°, 24.4° and 28.9° (Figure 6.3c). The PXRD patterns obtained for CAPE-CAF, CAPE-INC and CAPE-NIC, different from that of the parent compound and coformers confirms cocrystallization of CAPE with coformers forming new solid phase.

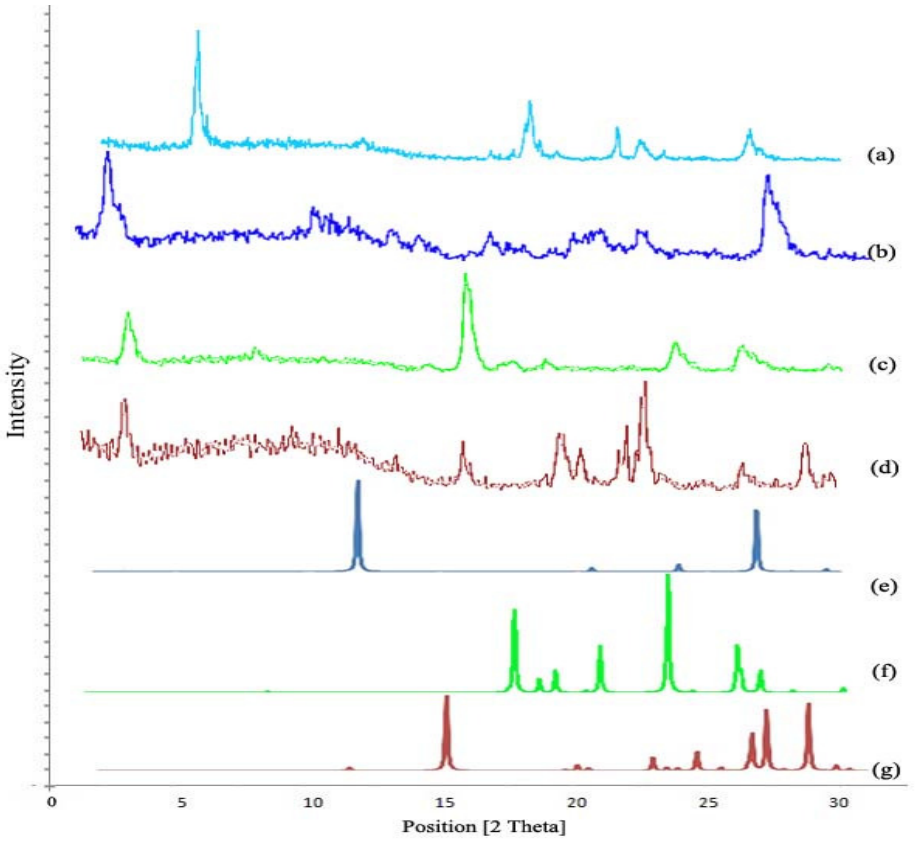


Figure 6.3. Experimental Powder diffraction patterns of a) pure CAPE; b) CAPE-CAF; c) CAPE-INIC; d) CAPE-NIC; e) CAF f) INIC and g) NIC

Raman spectroscopy:

Cocrystallization has significant impact on the solid state of participating molecules, primarily altering their hydrogen bond patterns. This would have major influence on the vibrational states of their functional groups, which can be detected through spectroscopic technique such as Raman spectroscopy (Fevotte, 2007, Soares and Carneiro, 2013). CAPE and its cocrystals were characterized by Raman spectroscopy in order to ascertain the influence of solid state modification on the vibrational states of participating atoms and differentiate the novel crystalline phases from the starting materials.

Figures 6.4A, 6.4B and 6.4C described Raman spectra of CAPE-NIC, CAPE: INIC and CAPE-CAF in comparison to its starting materials. In the raman spectrum of CAPE, the ester C=O stretch occurs at 1680.55 cm^{-1}, the asymmetric and symmetric C=C stretch

occurs at 1598.7 and 1451.8 cm^{-1} and the C-O stretch occurs at 1156.5 cm^{-1}. Similarly in the raman spectrum of NIC, the N-H stretch occurs at 3368.9 cm^{-1}, the C=O at 1676.39 cm^{-1} and C-O at 1159.3 cm^{-1}. On forming cocrystal, the amide C=O of NIC undergoes a red shift to 1634.7 cm^{-1} indicating the enhanced single bond character of the C=O group on forming a hydrogen bond with the phenolic O-H of CAPE. The other cocrystals where crystal structures are not available currently, changes in their vibrational patterns may be explained through raman spectrum. In pure INIC, the N-H stretch occurs at 3357.8 cm^{-1} and the C=O at 1676.3 cm^{-1}. In the cocrystal, the amide C=O undergoes a blue shift to 1697.1 cm^{-1} and C-O to 1163.4 cm^{-1}. In pure CAF, the C=O appears at 1695 cm^{-1} which undergoes a red shift to 1687.5 cm^{-1} in the cocrystal. Thus, the raman spectra ably complements other characterization techniques in establishing the formation of novel crystalline phases.

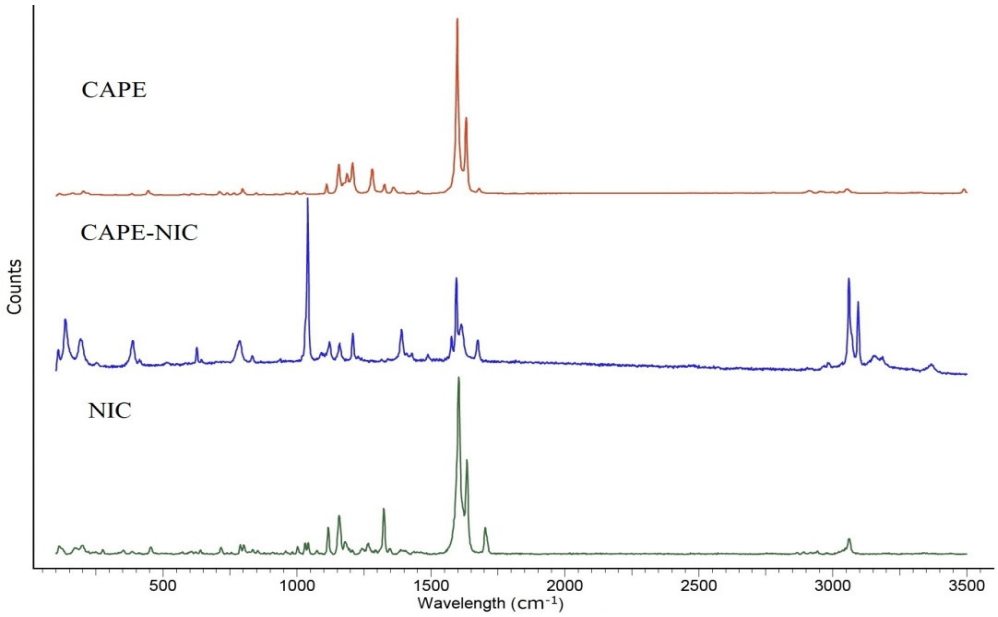


Figure 6.4A. Raman spectrum of CAPE-NIC in comparison to its starting materials

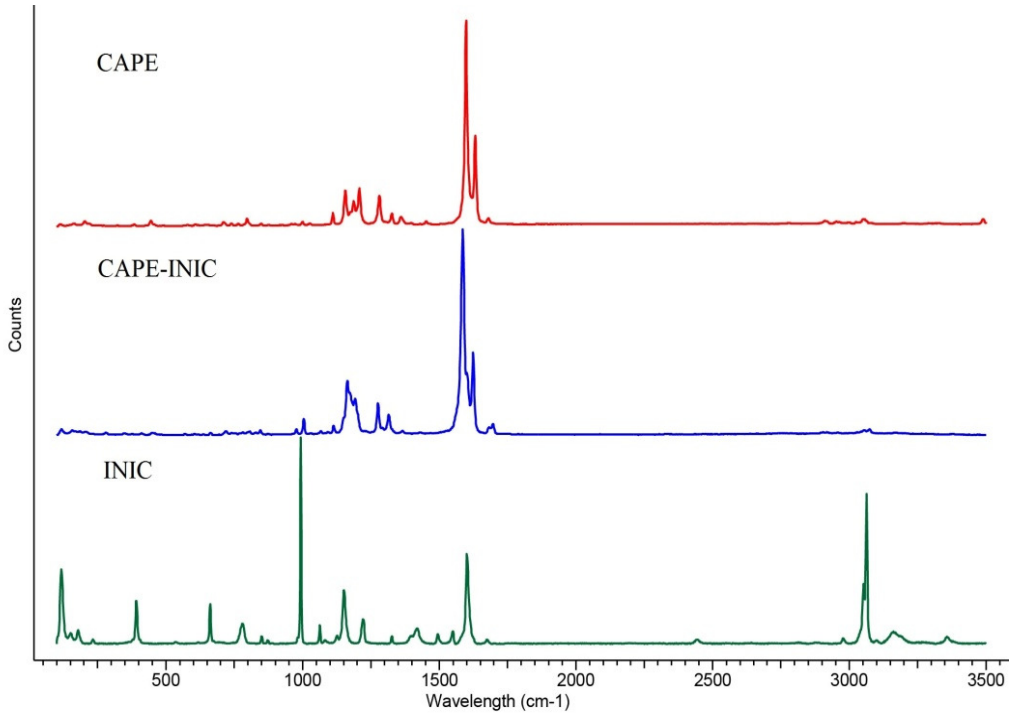


Figure 6.4B. Raman spectrum of CAPE-INIC in comparison to its starting materials

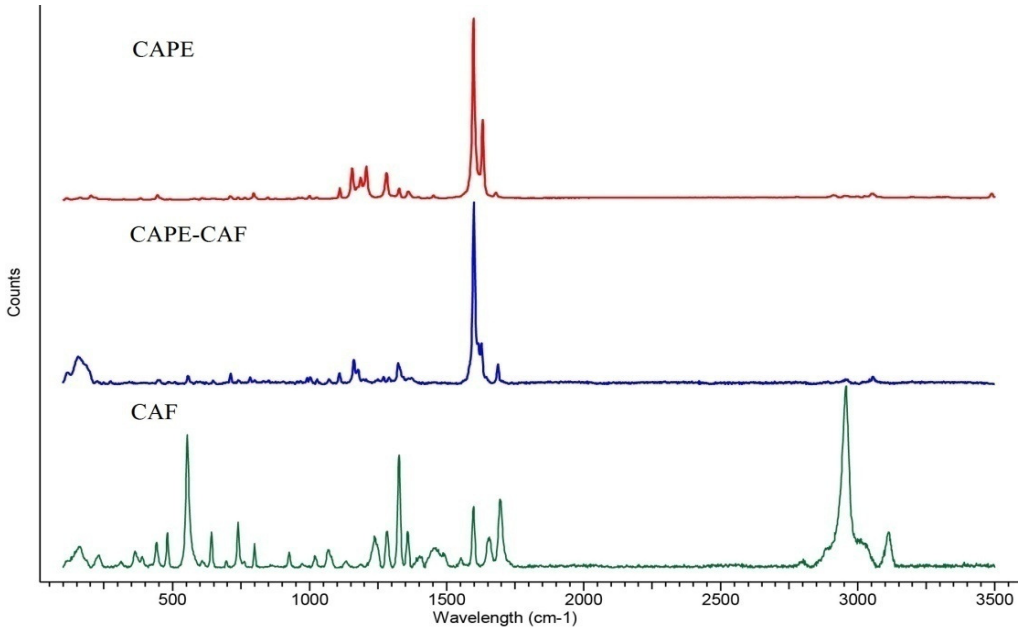


Figure 6.4C. Raman spectrum of CAPE-CAF in comparison to its starting materials

Equilibrium Solubility:

The primary objective of developing cocrystals for CAPE was to overcome its poor solubility which hinders its bioavailability thereby limiting its efficacy. The objective was designed based on the hypothesis that the high solubility coformers would alter the hydrogen bond patterns in the CAPE which would improve its aqueous solubility envisaging enhancement in bioavailability. Equilibrium solubility experiments on CAPE and its cocrystals were performed in distilled water and the samples were analyzed by HPLC. These experiments were performed for 24 hrs by making a saturated solution of the compounds in pure water. Figures 6.4A, 6.4B, 6.4C and 6.4D depict HPLC chromatograms obtained for CAPE, CAPE-CAF, CAPE-INIC and CAPE-NIC cocrystal samples respectively. The chromatograms demonstrated different retention times (Rt min ± SD) for CAPE (3.702 ± 0.02), CAF (3.123 ± 0.03), INIC (2.945 ± 0.02) and NIC (3.006 ± 0.01) (Figure 6.5A-D).

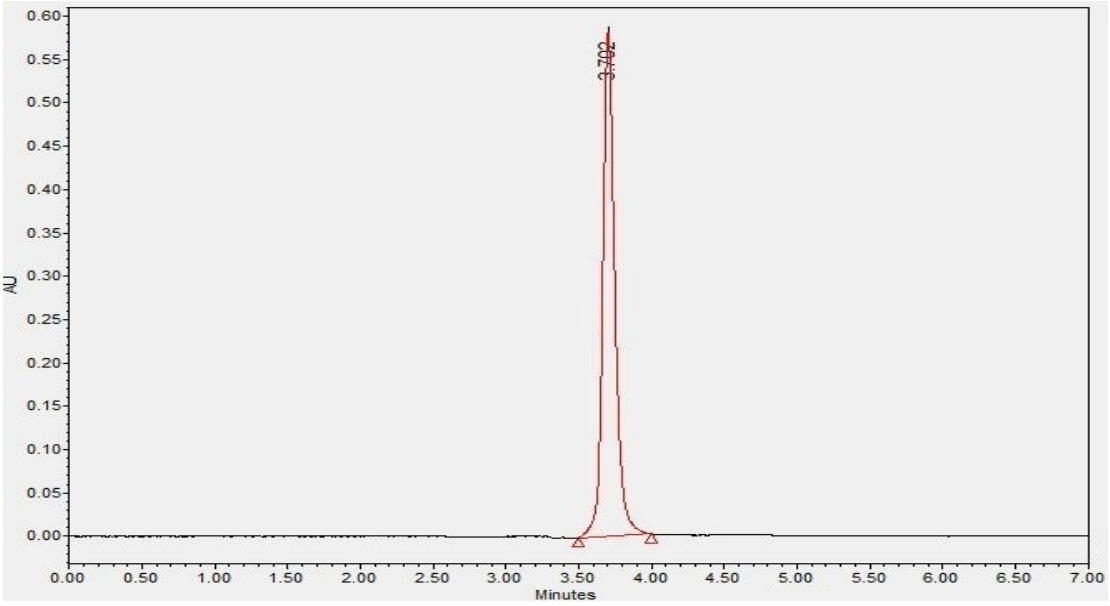


Figure 6.5A. Representative HPLC chromatogram for CAPE.

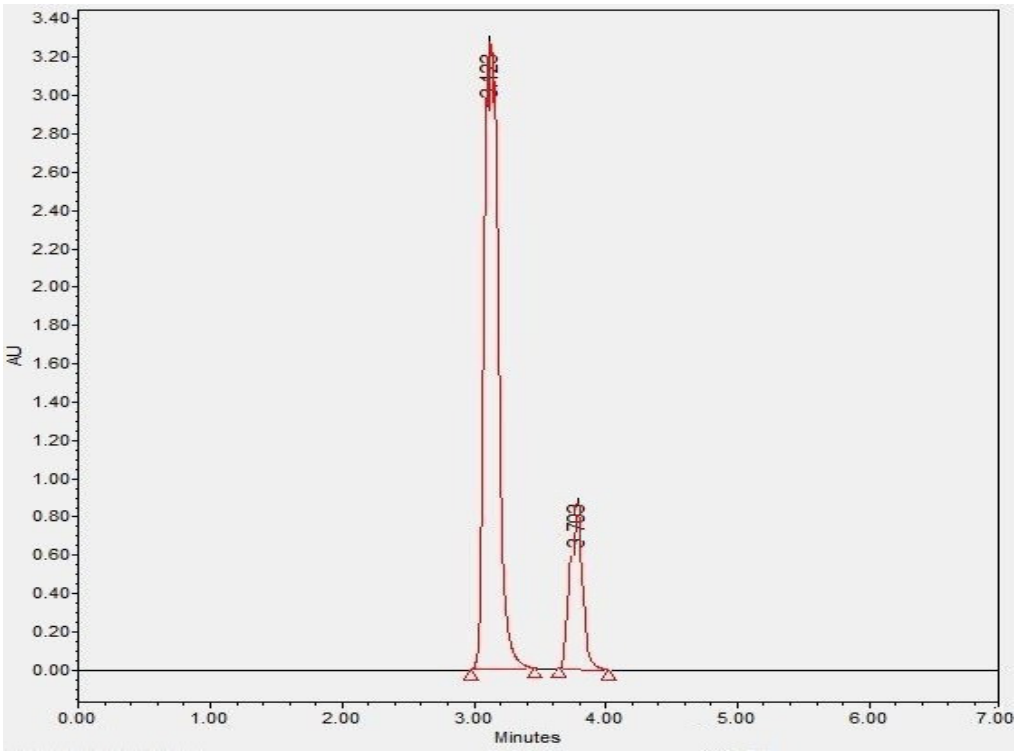


Figure 6.5B. Chromatogram for CAPE-CAF. The chromatogram depicts elution at 3.123 min for CAF and 3.703 min for CAPE

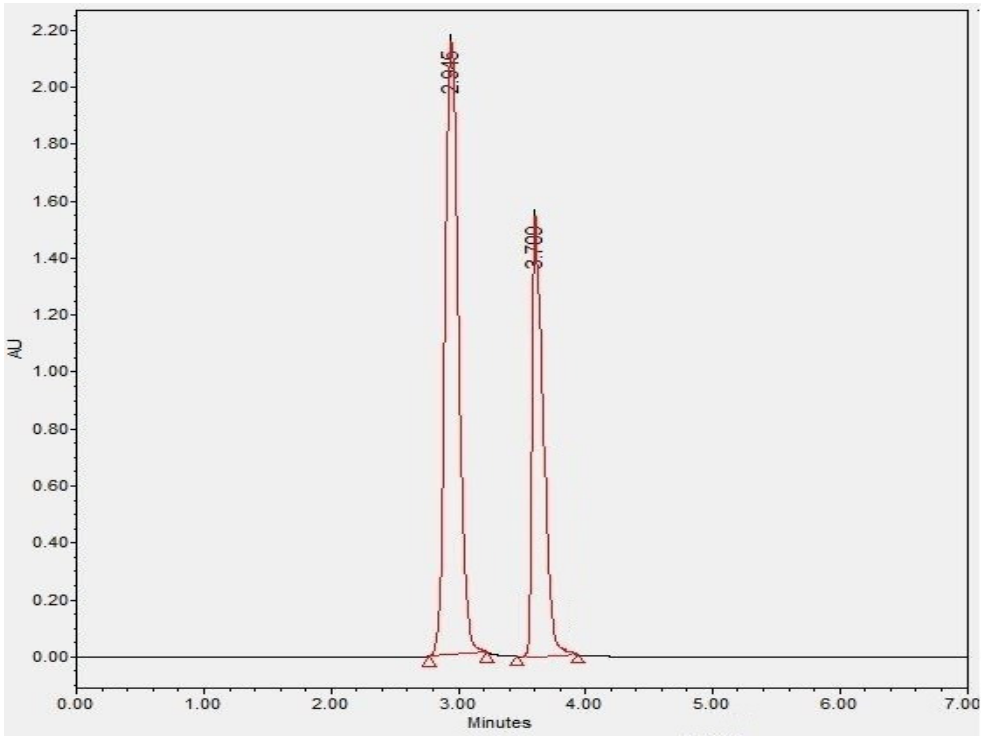


Figure 6.5C. Chromatogram for CAPE-INIC. The chromatogram depicts elution at 2.915 min for INIC and 3.70 min for CAPE

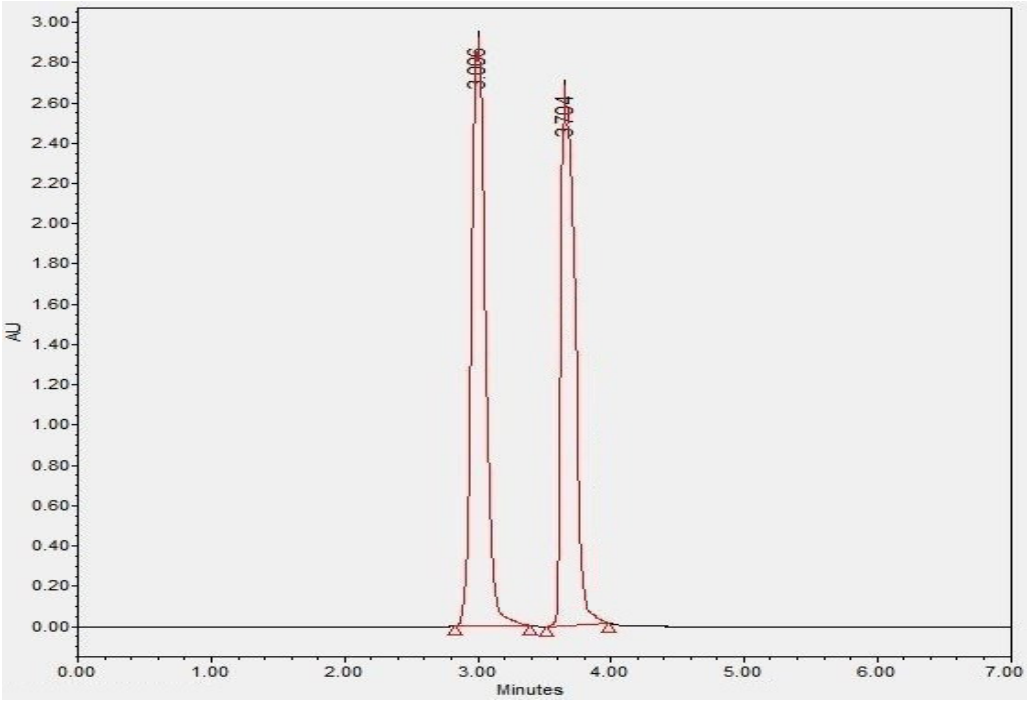


Figure 6.5D. Chromatogram for CAPE-NIC. The chromatogram depicts elution at 3.006 min for NIC and 3.704 min for CAPE

Table 6.4. Equilibrium solubility values of CAPE and its cocrystals

Compound	Equilibrium Solubility (mg/ml)	No. of times increment as compared to CAPE	Melting point of cocrystal (°C)
CAPE	0.021 ± 0.0013	-	129
CAPE-CAF	0.115 ± 0.0011	5.5	113.48
CAPE-INIC	0.158 ±0.0054	7.5	107.98
CAPE-NIC	0.371 ±0.0052	17.7	106.67

Table 6.4 describes the equilibrium solubility values determined for the CAPE and its cocrystals. The solubility experiments, apart from confirming the poor aqueous solubility of CAPE (0.021 ± 0.0013 mg/ml), highlighted the advantage in forming its cocrystals where the CAPE-CAF, CAPE-INIC and CAPE-NIC exhibited about 5.5, 7.5 and 17.7 times higher solubility as compared to CAPE. Interestingly, the solubility profile of CAPE cocrystals is inversely related to their melting point. The lower melting CAPE-NIC exhibited higher solubility followed by CAPE-INIC and CAPE-CAF.

Apart from ascribing to the higher solubility documented for nicotinamide in water (Etter, 1990, Etter, 1991), significant improvement in the aqueous solubility of CAPE-NIC may be understood through the BFDH (Bravais, Friedel, Donnay and Harker) model of CAPE-NIC crystal structure which describes the equilibrium morphology of a crystal. According to the model (Figure 6.6A), the hydrophilic -diol groups of CAPE project out of the major (100) face of the CAPE-NIC crystal structure burying the hydrophobic skeleton inside, enabling favorable interaction with the water molecules and thus increasing its solubility.

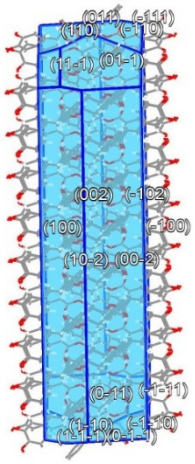


Figure 6.6A: BFDH model of CAPE-NIC showing the projected diol groups long the major (100) face. (Projected parallel to the (100) face)

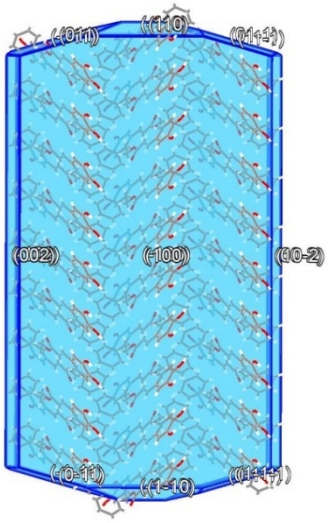


Figure 6.6B: BFDH model of CAPE-NIC showing the projected diol groups long the major (100) face. (Projected perpendicular to the (100) face)

Single crystal X-Ray Crystallography of CAPE-NIC:

Among the novel crystalline phases obtained for CAPE in the form of cocrystals, the single crystal structure of CAPE-NIC was determined by SC-X-ray crystallography. The structural parameters of CAPE-NIC are shown in table 6.5 and the hydrogen bond parameters are listed in table 6.6.

Table 6.5. Crystallographic Parameters of CAPE-NIC cocrystal

Emp form.	$C_{23}H_{22}N_2O_5$
Form wt	406.43
Cryst syst	Monoclinic
Sp gr	$P2_1/c$
T (K)	298(2)
a (Å)	22.0039(15)
b (Å)	5.2382(4)
c (Å)	17.9644(16)
α (º)	90
β (º)	99.975(8)
γ (º)	90
Z	4
V (Å^3)	2039.3(3)
Total no. of rflns.	6455
Unique rflns.	3457
Obsd. rflns.	2391
Parameters	287
R_1	0.0500
wR_2	0.1470
GOF	1.021

Table 6.6. Hydrogen bond distances and angles in CAPE-NIC cocrystal (neutron-normalized N–H 1.009 Å, O–H 0.983 Å, and C–H 1.083 Å distances)

D–H···A	D···A (Å)	H···A (Å)	D–H···A (°)	symmetry code
CAPE–NIC (1:1)				
O1–H1···O2	2.707(2)	2.19	115	--a
O1–H1···O5	2.790(3)	1.96	148	1–x, 1–y, –z
O2–H2···N3	2.708(3)	1.74	165	x, 3/2–y, –1/2+z
N2–H2A···O5	3.088(3)	2.29	150	x, –1+y, z
N2–H2B···O1	3.068(3)	2.58	114	1–x, –y, –z
N2–H2B···O2	3.005(3)	2.26	137	1–x, 1–y, –z

--a – Intramolecular hydrogen bonding

Crystal Structure Analysis of CAPE-NIC:

The 1:1 stoichiometric cocrystal crystallized in monoclinic $P2_1/c$ space group. The two-point heteromeric interaction between the 1,2-benzenediol moiety of CAPE and the amide of NIC (O1–H1···O5, 1.96Å, 148°; N2–H2B···O2, 2.26Å, 137°) is the prominent H-bonding synthon in this cocrystal. (Figure 6.7) The *syn* amide N-H forms a bifurcated H-bond with the hydroxyl oxygen of adjacent CAPE molecule, connecting the heteromeric pairs. Further, the O2 hydroxyl of CAPE forms O–H···N (O2–H2···N3, 1.74Å, 165°) H-bond with the pyridine N of NIC from adjacent layer completing the 3-dimensional packing (Figure 6.8).

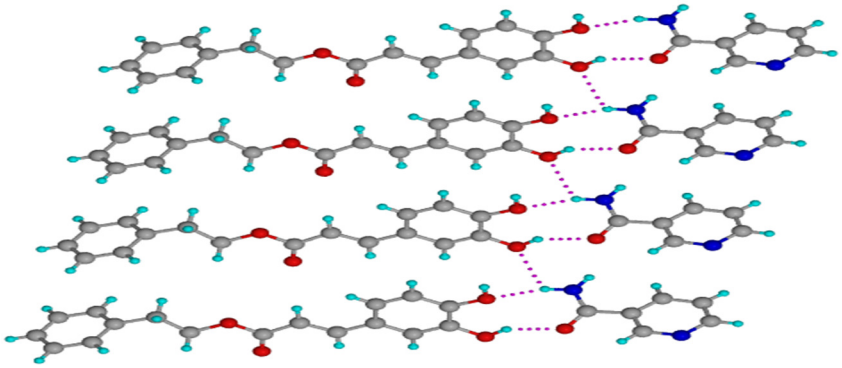


Figure 6.7. Heteromeric two point synthon within CAPE-NIC (CAPE molecules are H-bonded through heteromeric two point synthon with NIC molecules which are further interconnected through N-H···O H-bonds.)

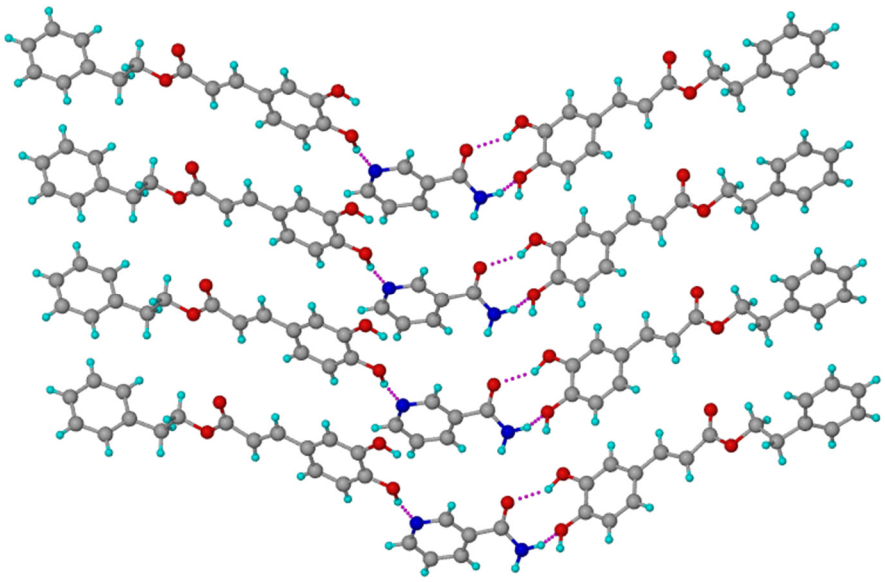


Figure 6.8. Adjacent layers of heteromeric CAPE-NIC pairs (these are connected through O-H···N H-bonds).

The heterosynthon between CAPE and NIC is relatively rare interaction as compared to their respective homo and competing heterosynthons. A Cambridge Structural Database (CSD) search for cocrystals with 1,2-benzenediol and amide functional group lends credence to this statement (Table 6.7). A CSD search (version 5.36, May 2015 update) with these functional groups on chemically different molecules resulted in 27 hits. Of these 17 crystal structures were stabilized by competing heterosynthons like acid-amide etc., 5 of these structures propagate through competing homosynthons like amide-amide, OH···OH etc. Only the remaining five structures form the 1,2-benzenediol and amide heterosynthon. The predominance of crystal structures stabilized by competing heterosynthons indicates the diol-amide heterosynthon as a much weaker interaction. In the CAPE-NIC structure, the diol-amide heterosynthon was favored due to the absence of other functional groups and preferred over the competing homosynthons.

Table 6.7. Refcodes with 1,2-benzenediol and amide functional groups in the same crystal structure

EVIJUO	FIXROV	GAZWUB	HEDRAL	HEDREP
HEDRIT	HEDROZ	HUMJII	MUPMOA	MUPNAN
MUPNUH	MUPPAP	NAXHOL	PEFGEO	PEFGEO01
PEFGEO02	PEFGEO03	PEFGIS	REBXIH	VEQTIW
ZEBXEL	ZEBXIP	ZEBXOV	ZEBXUB	ZIKNOY
ZIKPAM	ZIKPUG			

The refcodes marked in RED represent crystal structures with benzene diol and amide heterosynthon

The refcodes marked in BLUE represent crystal structures with competing homosynthons such as amide-amide, OH-OH etc

The refcodes marked in BLACK represent crystal structures with competing heterosynthons like acid-amide, amide-pyridine etc.

6.3.: Conclusion

The study confirms successful formation of novel crystalline phases for CAPE in the form CAPE-CAF, CAPE-INIC and CAPE-NIC cocrystals by employing microwave assisted cocrystallization technique. Results of PXRD and DSC confirm synthesis and phase purity of CAPE-CAF, CAPE-INIC and CAPE-NIC cocrystals. Findings of Raman spectra for developed cocrystals complemented these results in conferring the formation of new crystalline phases of CAPE. Interestingly, the architecture of CAPE-NIC cocrystal as revealed by SC-X-Ray crystallography demonstrated stabilization of crystal structure by rarely preferred 1,2-benzenediol and amide heterosynthon. Cocrystallization of CAPE with CAF, INIC and NIC evidently improved its aqueous solubility by 5.5, 7.5 and 17.7 times respectively, envisaging possible improvement in its biopharmaceutical performance and therapeutic efficacy.

References:

Almarsson O, Zaworotko MJ (2004). Crystal engineering of the composition of pharmaceutical phases. Do pharmaceutical cocrystals represent a new path to improved medicines? *Chemical Communications*, 1889-1896.

Blagden N, de Matas N, Gavan PT, York P (2007). Crystal engineering of active pharmaceutical ingredients to improve solubility and dissolution rates. *Advanced Drug Delivery Reviews*, 59, 617-630.

Ceschel G, Maffei P, Sforzini A, Borgia SL, Yasin A and Ronchi C (2002). In vitro permeation through porcine buccal mucosa of caffeic acid phenetyl ester (CAPE) from a topical mucoadhesive gel containing propolis. *Fitoterapia,* 73**:**S44-S52.

Good DJ and Rodriguez HN (2009). Solubility advantage of pharmaceutical cocrystals, *Crystal Growth Design*, 9, 2252-2264

Guidance for Industry: Regulatory Classification of Pharmaceutical Co-Crystals (2013). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), USA.

Datta AK (2001). Handbook of microwave technology for food application. CRC Press.

Etter MC (1990). Encoding and decoding hydrogen-bond patterns of organic compounds. *Accounts of Chemical Research,* 23**:**120-26.

Etter MC (1991). Hydrogen bonds as design elements in organic chemistry. *The Journal of Physical Chemistry,* 95**:**4601-10.

Fevotte G (2007). In situ Raman spectroscopy for in-line control of pharmaceutical crystallization and solids elaboration processes: a review. *Chemical Engineering Research and Design,* 85**:**906-20.

Goud NR, Gangavaram S, Suresh K, Pal S, Manjunatha SG, Nambiar S and Nangia A (2012). Novel furosemide cocrystals and selection of high solubility drug forms. *Journal of pharmaceutical sciences,* 101**:**664-80.

Hong C, Xie Y, Yao Y, Li G, Yuan X and Shen H (2015). A Novel Strategy for Pharmaceutical Cocrystal Generation Without Knowledge of Stoichiometric Ratio: Myricetin Cocrystals and a Ternary Phase Diagram. Pharmaceutical research, 32:47-60

Jones W, Motherwell W and Trask AV (2006). Pharmaceutical cocrystals: an emerging approach to physical property enhancement. *MRS bulletin,* 31**:**875-79.

Karki S, Fabian L, Friscic T and Jones W (2007). Powder X-ray diffraction as an emerging method to structurally characterize organic solids. Organic Letters, 9 (16): 3133-3136

Karki S, Friscic T, Fabian L, Laity PR, Day GM and Jones W (2009). Improving mechanical properties of crystalline solids by cocrystal formation: new compressible forms of paracetamol. *Advanced Materials*, 21 (38-39), 3905-3909

Kappe CO (2004). Controlled microwave heating in modern organic synthesis. *Angewandte Chemie International Edition,* 43**:**6250-84.

Norby P (2006). In-situ XRD as a tool to understanding zeolite crystallization. *Current opinion in colloid and interface science,* 11**:**118-25.

Pagire S, Korde S, Ambardekar R, Deshmukh S, Dash RC, Dhumal R and Paradkar A (2013). Microwave assisted synthesis of caffeine/maleic acid co-crystals: the role of the dielectric and physicochemical properties of the solvent. *CrystEngComm,* 15**:**3705-10.

Qiao N,Li M, Schlindwein W, Malek N, Davies A,Trappitt G (2011). Pharmaceutical cocrystals: An overview, *International Journal of Pharmaceutics*, 419, 1-11.

Sanphui P, Goud NR, Khandavilli UR and Nangia A (2011). Fast dissolving curcumin cocrystals. Crystal Growth and Design, 11:4135-45

Schultheiss N and Newman A (2009). Pharmaceutical cocrystals and their physicochemical properties. *Crystal Growth and Design*, 9 (6), 2950- 2967.

Shan N and Zaworotko MJ (2008). The role of cocrystals in pharmaceutical science. *Drug discovery today,* 13**:**440-46.

Smith AJ, Kavuru P, Wojtas L, Zaworotko MJ and Shytle RD (2011). Cocrystals of quercetin with improved solubility and oral bioavailability. Molecular pharmaceutics, 8:1867-76.

Smith AJ, Kavuru P, Arora KK, Kesani S, Tan J, Zaworotko MJ and Shytle RD (2013). Crystal engineering of green tea epigallocatechin-3-gallate (EGCg) cocrystals and pharmacokinetic modulation in rats. Molecular pharmaceutics, 10:2948-61.

Soares FLF and Carneiro RL (2013). Green Synthesis of Ibuprofen–Nicotinamide Cocrystals and in-line evaluation by raman spectroscopy. *Crystal Growth*, 13, 1510–1517.

Trask AV and Jones W (2005). Crystal engineering of organic cocrystals by the solid-state grinding approach. Organic solid state reactions. Springer, 41-70.

Trask AV, Motherwell WDS and Jones W (2006). Physical stability enhancement of theophylline via cocrystallization. *International Journal of Pharmaceutics*, 320 (1-2), 114-123

Vishweshwar P, McMahon JA, Bis, JA, Zaworotko MJ (2006). Pharmaceutical Co-crystals. *Journal of Pharmaceutical Sciiences.* 2006, 95, 499-516.

Walsh RB, Bradner M, Fleischman S, Morales L, Moulton B, Rodriguez-Hornedo N and Zaworotko M (2003). Crystal engineering of the composition of pharmaceutical phases. *Chemical Communications***:**186-87.

Chapter 7. Summary and Conclusion

This chapter will summarize all the study results and provide conclusions. Further it will discuss the scope for future developments in the field of apiproducts.

7. SUMMARY AND CONCLUSION

7A. Summary:

The studies described in this dissertation deal with exploration and potentiation of nutritional and therapeutic competency of multicomponent active apiproducts viz. bee pollen and propolis. In recent years, functional foods or processed foods, (which exerts beneficial effect in one or more physiological functions, increases well-being and / or decreases the risk of suffering from a particular medical condition) have attracted major focus of research in natural products. Apiproducts, such as bee pollen, propolis on account of their richness in antioxidant principles especially the polyphenols and flavonoids, find wide applications in food science. Apart from functional foods, these apiproducts may also serve as an important source of therapeutically active ingredients. In this context we have initiated explorations on processing of bee pollen and propolis using pharmaceutically acceptable excipients thereby converting them into potentiated or fortified value added apiceuticals. Further fundamental study on single active component (CAPE) originating from propolis was performed to generate innovative crystalline forms of CAPE as cocrystals to improve its aqueous solubility which would direct improvement in its biopharmaceutical and therapeutic performance. Developed CAPE cocrystals with improved solubility can also serve as active apiceutical ingredient.

The studies were summarized as follows:

I. Bee Pollen: Exploration & potentiation of nutraceutical competence:

A. Investigation of nutraceutical potential of monofloral Indian mustard bee pollen (MIMBP)

The nutrient composition of MIMBP in terms of total protein, fat, carbohydrate, moisture, ash content along with energy value were determined. Further the MIMBP extract was screened for determination of total polyphenol and flavonoid content. A simple, specific and accurate HPLC-PDA method for identification and quantification of free flavonoid aglycones from the MIMBP sample was developed. The method was validated for parameters linearity, precision, specificity, accuracy, and robustness in order to ascertain the suitability of method for analysis of flavonoid aglycone markers. Further the MIMBP

was screened for *in vitro* antioxidant activity in terms of radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by spectroscopic method. The MIMBP was found to be comprised of proteins (18.22 ± 0.59 g/100g), fats (13.77 ± 0.68 g/100g), and carbohydrates (56.06 ± 1.74 g/100g), which result in its high energy value (421.09 ± 1.61 Kcal/100g). MIMBP was found to contain polyphenols (1828.61 ± 37.4 mg GAE/100g) and flavonoids (122.35 ± 5.31mg QE/100g). The HPLC-PDA analysis revealed the presence of kaempferol (6.54 ± 0.05 mg/100gm) and quercetin (5.14 ± 0.04 mg/100g) in MIMBP, which can be used as markers for determining the quality of bee pollen. The MIMBP extract showed DPPH free radical-scavenging activity with a half maximal inhibitory concentration of 54.79 µg/mL. The study thus demonstrated nutraceutical candidature of MIMBP. The study outcome will be useful to set national pollen standards for monofloral bee pollen as an initial attempt in establishing quality criteria for bee pollen worldwide.

B. Potentiation of MIMBP: Processing with edible lipid surfactant combinations followed by preclinical investigations.

MIMBP was processed with an edible lipid-surfactant mixtures to increase the extraction of polyphenols and flavonoid aglycones as analyzed by UV spectroscopy and HPLC-PDA method. The lipids screened for study include Captex 355, Lauroglycol FCC and Labrafil M 1944 in combination with surfactant Tween 80. MIMBP and the processed pollen (PMIMBP) were subjected to scanning electron microscopy. Further both the MIMBP and PMIMBP were subjected to *in vitro* gastric digestion protocol so as to determine the bio-accessibility of polyphenols. The MIMBP and PMIMBP were further subjected to preclinical investigations starting with acute oral toxicity studies. Further their effect on swimming exercise-induced oxidative stress muscular implications in Wistar rats was studied. The rats in different groups were fed with MIMBP or PMIMBP supplements at a dose of 100 mg/kg, 200 mg/kg and 300 mg/kg individually, while being subjected to chronic swimming exercise for 4 weeks (5 days/week). Various biochemical [superoxide dismutase (SOD), glutathione (GSH), malonaldehyde (MDA), nitric oxide (NO), and total protein content], mitochondrial (Complex I, II, III, and IV enzyme

activity), and molecular (myostatin mRNA expression) parameters were monitored in the gastrocnemius muscle of each group.

MIMBP processed with Captex 355:Tween80 [at weight (mg) ratio 500:750 per g of pollen] system achieved highest extraction of total polyphenols (3123.35 ± 163.44 mg/ 100g) and flavonoid aglycones [kaempferol (8.89 ± 0.31 mg/100g) and quercetin (13.02 ± 0.25 mg/100g)]. SEM analysis confirms effective rupture of the exine part of PMIMBP which was ascribed to combined effect of lipid-surfactant treatment and process of vigorous trituration leading increased extraction of polyphenols.

The *in vitro* bio-accessibility study demonstrates 2.66 fold increase in the bio-accessible polyphenols content of PMIMBP when compared with that of MIMBP. The study further confirms effective breaking of the pollen wall for PMIMBP suggesting improved digestibility of same.

Administration of both MIMBP (300 mg/kg) and PMIMBP (100 mg/kg, 200 mg/kg, and 300 mg/kg) exerted an antioxidant effect by significantly modulating the SOD, GSH, MDA, NO, and total protein levels. Further MIMBP (300 mg/kg) and PMIMBP (200 mg/kg and 300 mg/kg) significantly improved impaired mitochondrial Complex I, II, III, and IV enzyme activity. Significant down-regulation of myostatin mRNA expression by MIMBP (300 mg/kg) and PMIMBP (200 mg/kg and 300 mg/kg) indicates their muscle protectant role in oxidative stress conditions. The study thus establishes antioxidant, mitochondrial up-regulatory and myostatin inhibitory effects of MIMBP. Further processing of MIMBP with an edible lipid-surfactant mixture was found to potentiate its antioxidant and therapeutic efficacy.

II. Propolis: Exploration of Green extraction Method

A. Development of HPLC-PDA method for simultaneous estimation of principal flavonoid marker components of propolis

HPLC-PDA method for quantitative determination of flavonoids caffeic acid, rutin, quercetin, apigenin, kaempferol, chrysin, acacetin, galangin and cape from propolis sample was developed. Accurate quantitation of the flavonoid compounds was

ascertained by detecting the individual compound at respective maximum wavelength of absorption (λmax) using Photo-diode array detector. The method was validated for the parameters linearity, precision, specificity, accuracy and robustness in order to ensure its suitability for analysis of propolis extracts.

B. Microwave Assisted Extraction (MAE) of Propolis:

A green method to improve microwave assisted extraction (MAE) of flavonoids from propolis was developed using Gelucire 44/14 as extractant. Propolis was initially subjected to temperature controlled closed-vessel MAE using ethanol as conventional organic solvent. The MAE conditions were optimized to achieve maximum extraction of flavonoids by varying the process time and temperature. Alternatively propolis was extracted using Gelucire 44/14 aqueous solutions of different concentrations (10 to 50% w/w) at the optimized set of conditions in MAE. In order to understand the process of Gelucire 44/14 based MAE, microwave absorptive properties of the Gelucire 44/14 solutions in terms of dielectric constant (ε'), dielectric loss (ε") and dissipation factor (Tan δ) were studied using open-ended coaxial-line probe and correlated with extraction of flavonoids.

Conventional MAE of propolis using ethanol as solvent achieved highest extraction of total flavonoids (62.201 ± 1.372 mg/g) in terms of screened flavonoid marker compounds at operating conditions of 65 °C for 20 min. The Gelucire 44/14 solution of concentration 40% w/w achieved highest extraction of total flavonoids (94.854 ± 1.589 mg/g) at same operating conditions. This confirms an ability of Gelucire 44/14 as extractant to improve MAE of propolis constituents.

The study of dielectric properties of Gelucire 44/14 solutions demonstrated that at a given temperature, the concentration of Gelucire 44/14 has profound effect on dielectric properties (ε') and (ε"). Incorporation of Gelucire 44/14 reduced the dielectric constant of water gradually. Observed reduction in ε' of water by addition of Gelucire 44/14 suggests interruption of tetrahedral structure of water molecules thereby reducing its polarity. Correlation of dielectric constant values with the flavonoids extraction yield demonstrates critical concentration range for Gelucire 44/14 solutions (30 to 40 % w/w)

wherein significant changes in ε' and flavonoids extraction yield were observed. This reduces the polarity of water to a degree favorable for facilitating solubilization of flavonoids thereby increasing the extraction yield at 40 % w/w of Gelucire 44/14. Further incorporation of Gelucire 44/14 in water gradually increased the Tan δ values of solution. Rising Tan δ values indicate improved conversion of absorbed microwave energy into thermal energy. The increased heat generation contributes to elevate the temperature of solution where maximum extraction of flavonoids was observed. The micro-structural interaction of Gelucire 44/14 with water molecules was studied by subzero temperature DSC analysis. Significant rise in the enthalpy (-26.84 Jg^{-1}) of solution at Gelucire 44/14 concentration 40 % w/w indicates stronger association of water molecules with the surfactant chains. This suggests effective interruption of tetrahedral structure of water molecules thereby reducing the dielectric constant of water. The study thus recognizes Gelucire 44/14 as promising extractant in MAE of flavonoids from propolis.

III. Caffeic acid phenethyl ester (CAPE): Development of cocrystals

The study involved application of crystal engineering principles to the active moiety of propolis 'CAPE', for development of cocrystals using pharmaceutically acceptable coformers. The cocrystallization was achieved by microwave assisted crystallization technique.

The synthesis of cocrystal was initiated with selection of coformer as the first step. Based on the potential sites for hydrogen bonding from the CAPE structure, coformers possessing complementary functional groups capable of forming hydrogen bonds with CAPE were selected. Individually equimolar quantities of CAPE and respective co-formers were mixed in separate glass tubes. To the individual mixtures, 60 % (w/v) of solvent was added and subjected to microwave irradiation in microwave reactor to induce cocrystallization. Upon completion of microwave irradiation protocol, obtained solid mass was subjected to PXRD and DSC analysis to ascertain the formation of cocrystal. CAPE was successfully found to get cocrystallized with coformers Caffeine (CAF), Isonicotinamide (INIC), Nicotinamide (NIC). Findings of Raman spectra for developed

cocrystals complemented the PXRD and DSC results in conferring the formation of crystal of CAPE. Interestingly, it was observed that all the cocrystal phases exhibited lower melting point as compared to the parent CAPE and the respective coformer. The cocrystal melting points showed a direct dependence on the coformer melting point, i.e. higher melting cocrystal was formed by a higher melting conformer. The obtained cocrystals were found to exhibit phase purity as evident from DSC findings. Further unique diffraction patterns for CAPE cocrystal in comparison to their starting materials were observed by PXRD analysis. Equilibrium solubility experiments showed that CAPE-CAF, CAPE-INIC and CAPE-NIC showed 5.5, 7.5 and 17.7 times higher solubility as compared to the parent compound. Interestingly, the architecture of CAPE-NIC cocrystal revealed by X-Ray crystallography demonstrated stabilization of crystal structure by rarely preferred 1,2-benzenediol and amide heterosynthon. The study establishes formation of innovative crystal forms of CAPE in the form of cocrystal with improved aqueous solubility.

7B. Conclusion

- Present work establishes nutraceutical potential of Monofloral Indian Mustard Bee Pollen (MIMBP) by revealing its rich nutrient content in terms of carbohydrates, proteins, fats and antioxidant principles polyphenols and flavonoids.
- The work also reveals muscle protectant ability of MIMBP against exercise induced oxidative stress implications. The study explores antioxidant, mitochondrial up-regulatory and myostatin inhibitory effects of MIMBP suggesting its usefulness in effective management of exercise-induced muscular stress.
- Processing of MIMBP with an edible lipid-surfactant mixture was found to improve its polyphenols availability for exerting beneficial effects thereby potentiating therapeutic efficacy of pollen.

- The green extraction method in terms of Gelucire 44/14 based MAE was successfully developed for fortification of propolis in terms of bioactive flavonoids. Gelucire 44/14 was recognized as green extractant in MAE which can be screened for processing of allied apiproducts as well.
- Studies on single component system successfully achieved cocrystallization of CAPE with pharmaceutically acceptable coformers which evidently improved its aqueous solubility directing improvement in biopharmaceutical and therapeutic performance. The study develops innovative crystalline phases for CAPE in the form CAPE-CAF, CAPE-INIC and CAPE-NIC cocrystals.

Future prospects:

With the advent developments in recent years, the focus of biomedicine research would be shifting towards exploration of natural products from plant or animal origin. Apiproducts such as MIMBP and propolis have now been proved to be nutritionally enriched. Further combinatorial investigations involving phytochemical and biomedicinal aspects of such apiproducts can reconcile the conventional apitherapy with modern biomedicine. Current study establishes applicability of pharmaceutical excipients for fortification of apiproducts, similarly screening of different excipients for processing of allied apiproducts can generate variety of value added apiceuticals. Based on the evident improvement in therapeutic efficiency of PMIMBP over MIMBP, it is believed that the lipid surfactant composition of the matrix might have contributed to improve the bioavailability of pollen nutrients; however systematic studies to determine their levels circulating in the blood and available at the site of absorption need to be undertaken. The Gelucire 44/14 extract of propolis on account of its advantages envisage improved biopharmaceutical and therapeutic performance, therefore detailed investigations about same need to be performed. Similarly development of CAPE cocrystal documented improvement in its solubility which makes it potential target to be screened for different *in vitro* cell lines, *in vivo* models and clinical investigations pertaining to cancer or other diseases.

STANDARD OPERATING PROCEDURES:

POONA COLLEGE OF PHARMACY	STANDARD OPERATING PROCEDURE Oral Administration	SOP 1

Objective: To provide a standard operating procedure for oral administration.

Procedure:

1. Hold the rat by the nope in one hand thus making the animal to open its mouth.
2. Insert the feeding cannula (containing hypodermic stainless steel needle, bent by 300 around 1 cm distance from tip, gauge blunted at the tip to avoid any injury to the inner surface of mouth and esophagus which is attached to calibrated syringe).
3. Fill the required amount of solution/suspension in the syringe, remove air bubbles.
4. Gently push the feeding cannula through the intra-dental space and into the esophagus without rotating the feeding needle.
5. Gently push the piston of syringe to administer the accurate volume of solution/suspension.

Note: If accidentally the drug enters into lungs then hold the animal by tail and suspend in air. Give light pat on its back and try to expel the solution from the lung.

Prepared by **Checked and approved by**

(Sameer Ketkar) (Prof. K.R. Mahadik)

STANDARD OPERATING PROCEDURES:

POONA COLLEGE OF PHARMACY	STANDARD OPERATING PROCEDURE	SOP 2
	Procedure for organ weight and preserving organ for histopathology	

Objective: To provide standard operating procedure for weighing and preserving organ for histopathology examination.

Procedure:

1. Give the longitudinal incision to expose the visceral organ of the rat by using sharp scissors.
2. Dissect the organ required for histopathology examination and remove the fat and other adhering material.
3. Transfer the organ carefully by holding gently with a blunt forceps to normal saline solution to wash the organ, keep it on a tissue paper in order to soak the saline solution.
4. Immediately weigh the organ and transfer the organ to the bottle containing 10 % formalin solution. Repeat step 3 and 4 carefully for each organ.
5. Keep the organ of same animal in one bottle and label the bottle for identification. Change the formalin solution after each 24 hours.

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Prepared by **Checked and approved by**

(Sameer Ketkar) (Prof. K. R. Mahadik)

कामये दुःखतप्रानाम् ।
प्राणिनम् आर्तिनाशनम् ॥
सत्यमेव जयते
केन्द्रीय मधुमक्खी अनुसंधान एवं प्रशिक्षण संस्थान
CENTRAL BEE RESEARCH AND TRAINING INSTITUTE
खादी और ग्रामोद्योग आयोग
KHADI AND VILLAGE INDUSTRIES COMMISSION
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CBRTI/BOT/01/2009-10

January 21, 2012

To
Sameer S. Ketkar
Poona College of Pharmacy
Bharati Vidyapeeth University
PUNE

Sub: Identification of pollen samples -reg.

Sir,

Please refer to your letter dated 09/01/2012 for identification of pollen samples. The samples were analysed to find out the sources, findings are as follows.

Sample No.	Pollen source
1/WB/2012	- *Brassica* spp. (Mustard)

Yours faithfully,

Director

PUBLICATIONS

- Ketkar, S., S., Rathore, A.S., Kandhare, A.D., Lohidasan, S., Bodhankar, S.L., Paradkar, A.R., Mahadik, K.R. Alleviating exercise induced muscular stress using Neat and Processed bee pollen: oxidative markers, mitochondrial enzymes and myostatin expression in rats. Integrative Medicine Research. 2015. doi:10.1016/j.imr.2015.02.003; Elsevier Publications.
- Ketkar, S.S., Rathore, A.S., Lohidasan, S., Rao, L., Paradkar, A.R., Mahadik, K.R. Investigation of the nutraceutical potential of monofloral Indian mustard bee pollen. Journal of Integrative Medicine. 2014; 12(4):379-89. Elsevier Publications.

In process:

- Ketkar, S. S., Rathore, A.S., Pagire, S.S., Paradkar, A.R., Mahadik, K.R.. Improving microwave assisted extraction of flavonoids from propolis using Gelucire 44/14 as extractant: Correlative studies on dielectric properties
- Ketkar, S. S., Pagire, S.S., Goud, R., Nangia, A., Paradkar, A.R., Mahadik, K.R. Development of cocrystals for Caffeic acid Phenethyl ester: studies on co-crystal architecture and solubility implications

- Patent applied:
  Indian Patent Application [1040/MUM/2014] Solvent free method of extraction of herbals