A DISSERTATION ON

# FINGERPRINTING OF MEDICINAL PLANTS SPECIFIC HONEY

SUBMITTED TO THE DEPARTMENT OF BIOTECHNOLOGY

INTEGRAL UNIVERSITY, LUCKNOW

In partial fulfillmentFor the

Degree of Master of Sciencein Biotechnology

BY

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M.Sc. Biotechnology

(IV semester)

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Lucknow

UNDER THE SUPERVISION OF

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## CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ms. Samreen Khan** during the months Feb-June, 2022 reportedin the present thesis was under my Co-supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is **"Fingerprinting of Medicinal Plants Specific honey"** is therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of M.Sc. Biotechnology, Department of Biosciences, Integral University, Lucknow,(U.P).

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## To whom it may concern

This is to certify that **Ms. Samreen Khan,** a student of M.Sc. Biotechnology (2nd year/4th semester), Integral University Lucknow, has completed her four-month dissertation work entitled "**Fingerprinting of Medicinal Plants Specific honey**" successfully. She has completed this work at CSIR-CIMAP (Central Institute of Medicinal and Aromatic Plants), Lucknow, under the guidance of **Dr. Ratnasekhar CH**. The dissertation was a compulsory part of her M.Sc. Biotechnology degree.

I wish her good luck and a bright future.

**Dr. Snober S. Mir**

**Head of Department of Biosciences**

# DECLARATION

I hereby declare that the dissertation entitled **"FINGERPRINTING OF MEDICINAL PLANTS SPECIFIC HONEY"** submitted by me for the award of the degree of Master of Science in Biotechnology to Integral University, Lucknow is a bonafide work carried out under the guidance and supervision of **Dr. Ratnasekhar CH**, Scientist/Assistance Professor, Department of Phytochemistry at CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, Uttar Pradesh, India. Samreen Khan, I declare that the work for the dissertation has not been submitted, for the award of any other degree or diploma and the assistance received from other sources or people has been duly acknowledged. I further declare and also aware that in case of any discrepancies or irregularities related to project idea, data, data representation, writing, acknowledgements, citations found in the thesis will be my sole responsibility.

Samreen Khan

M.Sc. Biotechnology

(IV Semester)

2020-2022

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***SAMREEN KHAN***

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# ABBREVEATIONS

| | |
|---|---|
| Mg | Mili gram |
| Kg | Kilo gram |
| % | Percent |
| Nm | Nanometer |
| Cm | Centimeter |
| α | Alpha |
| β | Beta |
| Mm | Milimeter |
| ml | Mililitre |
| µl | Microlitre |
| Rpm | Revolutions per minute |
| ˚C | Degree Celcius |
| µm | Micrometer |
| HMF | Hydroxymethylfurfural |
| PH | Potential of Hydrogen |
| MIR | Mid-infrared |
| NIR | Near-infrared |
| GC-MS | Gas chromatography-mass spectrometry |
| FT-NIR | Fourier transfrorm-Near infrared |
| DD-SIMCA | Data Driven soft Independent Modelling of class Analogy |
| MATLAB | Matrix Laboratory |
| MSTFA | N-Methyl-N-(trimethylsilyl)trifluoroacetamide |
| TMCS | Trimethysiyl chloride |

# INTRODUCTION

# 1. INTRODUCTION

Honey is a natural material which is made by honey bees (*Apis mellifera L.)* from different plant secretions. Honey is made from flower nectar and has been used to heal a variety of ailments since antiquity. The nectar-producing plant type, bee species, geographic location, and harvesting conditions all influence the basic qualities of honey. Honey is divided into two categories based on the secretions of plants used in its production: The two types of honey are I blossom honey, which is produced from flower nectar, and (ii) honeydew honey, which is produced from plant secretions other than flower secretions or excretions of sucking insects. The botanical origin of the source of nectars or secretions determines the content and attributes of honey. (Bertelli et al., 2010), with carbohydrates being the most important elements. Honey is a sugary syrup with a high carbohydrate content. Honey is mostly made from floral nectars, with fructose and glucose as the primary constituents.

**Fig.1.1:** Honey image

According to the International Honey Organization's standards, honey is "the natural sweet substance produced by honey bees from plant nectar, secretions of living parts of plants, or excretions of plant-sucking insects on living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in the honey comb to ripen and mature (Codex alimentarius,2001)."The European Union's (EU) definition of honey is very similar, with the exception that it specifies Apis mellifera as the bee species (Directive 2014/63/EU).Honey is a popular natural product that is

valued not just because of its flavour and nutritional value, but also because of its health benefits. Since ancient times, honey has been used to cure wounds, burns, colds, and sore throats. Recent research have found that honey contains antibacterial,hepato-protective, hypoglycemic, antihypertensive, antifungal, anti-inflammatory, and antioxidant properties (Theunissen et al., 2001; Akbulut et al.; 2009, Erejuwa et al., 2012; Gomes et al.; 2010). Honey also has a high concentration of organic acids. Lactic, formic, butyric, tartaric, pyruvic, acetic, citric, oxalic, succinic, malic, maleic, -ketoglutaric, glucose-6-phosphate, pyroglutamic, and glycolic acids are only a few examples of these acids. The presence of enzymesdistinguishes honey from other sweetening agents, which is a unique feature. Yeasts, nectar, pollen, bees, and microbes are among the sources of these enzymes. When heated, honey can destabilise or disrupt these enzymes (Hebbar et al., 2003). Honeys with significant nectar or honeydew contributions from a variety of plant species are known as polyfloral or multifloral honeys. They're usually just referred to as "honey." Unifloral honey is unlikely to be generated by free-flying bees. Honey that comprises themajority of nectar or honeydew from a single plant species is referred to as unifloral honey. Honey's composition, flavour, and colour are all affected by the botanical source (Oddo et al., 2004).Honey is regulated by the Codex Alimentarius Standard and the European Union Council Directive on honey. Various articles (Oddo, Ruoff, and K) have documented the physical, chemical, and pollen analytical features of the most notable unifloral honeys (2004). Polyfloral honey, unlike unifloral honey, do not have unique physical or chemical properties apart from a great deal of diversity, making verification extremely difficult. Consumer demand for particular honey kinds has sparked interest in the development of unifloral honey, resulting in a commercial interest among beekeepers. Recent usage of particular honey kinds in therapeutic or technical applications may also explain the need for a trustworthy botanical origins determination.

**Traditional use of honey through the centuries**

A wall painting in Spain estimated to be about 10,000 years old depicts two human figures collecting honey from a bee nest. It is considered the earliest evidence of honey collection by humans (Allsop and Miller, 1996). Historically,

the medicinal use of honey has been recorded in the ancient age as early as 8000 years ago. The writings in Sumerian clay tablets dating back to 6200 BC, Vedas over 5000 years old, Egyptian papyri of 1900-1250 BC, many religious texts, and the works of Hippocrates.

Present study is on authenticity in honey and its adulteration is harmful to human health so this is a very important issue. Honey is a famous natural product, not only because of its sweetness and nutritive values, but also because of the health benefits it provides. Authentication and adulteration of honey has been a focus of fraudulent manufacture. Nowadays, authenticity and adulteration are major concerns. Authentication is a crucial aspect of food quality control, cleanliness, and safety. To ensure food safety and customer satisfaction, the detection and identification of food adulterants requires the development of unique and effective analytical methods for verification of composition, quality, and authenticity. Globally, authentication of honey production with the main issues related to sugar syrup addition and water content; and the labelled origin (geographical and/or botanical) and "organic" provenance. Fingerprinting in combination with DD SIMCA techniques is a powerful tool for detecting and controlling food fraud. Our idea was to create a Fingerprinting non-targeted method for authenticating commercial honey by FT-NIR, GC-MS, and analysis of FT-NIR data by MATLAB using DD SIMCA method. Based on the characteristics of honey, pure honey has been identified and its authenticity has been established. Different analytical approaches to test honey authenticity have been utilised to detect these honey frauds as it becomes a significant duty for processors, merchants, consumers, and regulatory authorities. This study examine all of these issues and focuses on techniques for identifying various forms of honey adulteration and authenticity. Because of the difficulty in authenticating honey and the prevalence of adulteration, numerous cutting-edge analytical techniques have been developed.

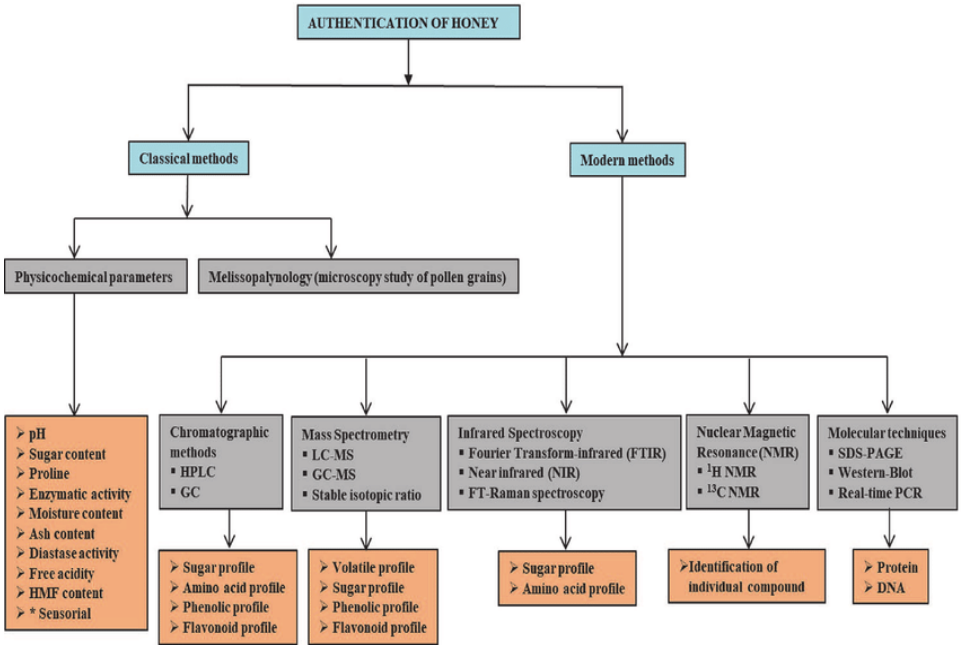



**Fig.1.2.** Conventional and Modern analytical methods used for honey authenticity

# OBJECTIVE

## 2. OBJECTIVEOBJECTIVE

The study was targeted to analyse the Fingerprinting by experimentation of

- Collection of honey material
- extraction of honey in ethanol solvent
- Fingerprinting of botanical origin of honey by :

✓ FT-NIR fingerprinting identifies botanical specific honey

✓ GC-MS analysis of honey samples

# REVIEW OF LITERATURE

# 3. REVIEW OF LITERATURE

A natural producer created from floral nectar by honeybees is called honey (*Apis mellifera*; family: Apidae) (Dashora et al., 2011). Honey has been used by humans since ancient, around 5500 years ago.

Honey is a sweet, viscous, amber-colored liquid made by honeybees. It is an internationally traded commodity that is sold under several brand names in India and around the world. India is one of the world's most biodiverse countries, with diverse ecosystems. Prevailing climatic conditions encouraged different honeybee species such as *Apis laboriosa*, *Apis dorsata*, *Apis florea*, *Apis cerana*, *Apis mellifera*, and stingless bee species such as *Trigona*, *Tetragonula*, and *Melipona* to produce unifloral and multifloral honey in temperate, tropical, and sub-tropical ecosystems both in hilly and plain areas of India. Southern Karnataka's diverse ecological conditions have generated a huge potential for unifloral and multifloral honey production.

Now, supermarket honey seem like a pretty uniform commodity, but the true honey varies a lot based on what the bees have been pollinating. Folks in the honey bees talks about monofloral and multifloral honey. Monofloral honey are the results of bees pollinating primarily one type of flower, such as Ajwain honey, Eucalyptus honey, neem honey, tulsi honey and Coriander honey etc. by setting up the hives in the fields of a specific type of flower during favourable period in every year. Each of these honeys comes from a different flower varies in colours and flavours. Generally bees forages in one mile radius around the hives if they migrates from their original place then it will give mix type of honey e.g. Multiforal honey which contains a mix of nectar sources hence mixed type of honey.

The term “polyfloral” and “multifloral” refers to honey that include significant nectar of honeydew input from numerous plant species. It is produced from the nectar of numerous floral species. Depending on the nectar source, it has a wide range of colour, flavour and potency. Additionally multifloral honey has antibacterial properties and its frequency used to treat allergies and anaemia. Regular consumption has a nourishing impact on the skin and can help with

condition of heart, stomach and intestines. Multifloral honey as mentioned above that is suitable for has a moderate flavour both adults and children's to ingest as a healthier alternative and sugars and sweetners. The word "honey" is usually all that is written on them. The term "unifloral honey" refers to honey that contains the majority of its nectar or honeydew from a single plant species. Honey's composition, flavour, and colour are all affected by the botanical source (Persano et al., 2004).

Currently, there is an increasing economic interest in the production of unifloral honeys. In fact, a lot of consumers enjoy having a wide selection of honey options and favour unifloral honeys over polyfloral honeys. Beekeepers can compete with low-cost polyfloral honey imported from other nations by producing unifloral honey. A reliable botanical origin assessment may also be required given the increased interest in the therapeutic or technical uses of specific honey varieties. According to the EU Council Directive (EU Council, 2002) and the Codex Alimentarius Standard for Honey (Codex Committee on Sugars, 2001), it is acceptable to apply a botanical label for honey if it primarily derives from the designated floral source. The most significant European unifloral honeys' physical, chemical, and pollen analytical characteristics have been described in numerous articles. (Crane et al., 1984; Moar et al.; 1985; Persano et al.; 1995; Piazza et al.; 2004).

## MEDICINAL USES OF HONEY

Honey's usage as a medication and an ointment is mentioned in the first recorded reference to honey, which dates back to 2100-2000 BC on a Sumerian tablet. When addressing different honeys, Aristotle (384-322 BC) described pale honey as "useful as a balm for sore eyes and sores." **Manuka** honey has been shown to have antibacterial activity against pathogenic bacteria including *Staphylococcus aureus* (S. aureus) and *Helicobacter pylori* (H. pylori), making it a viable functional food for woundand stomach ulcer treatment. Honey has been used to speed wound healing since ancient times, and the ability of honey to help in wound healing has been proved numerous times. Honey is becoming more widely accepted as a cure for ulcers, bed sores, and other skin diseases caused by burns and wounds.

Honey's healing properties are due to its antibacterial capabilities, ability to maintain a moist wound environment that promotes healing, and high viscosity, which helps to form a protective barrier against infection. Honey has been reported to be particularly successful as a treatment for wounds, burns, skin ulcers, and inflammations; honey's antibacterial characteristics help the wound heal faster by promoting the creation of new tissue. Honey has long been used as anti-aging, immune system enhancement, germ killing, bronchial phlegm therapy, and relief from a sore throat, cough, and cold. Honey also has anti-inflammatory (Akhmazillah et al., 2013), antioxidant (Saiful et al.,2016) According to the literature it shows anti-cancer effects against breast and cervical cancer (Kassim et al., 2012), prostate cancer (Rao et al., 2016), and osteosarcoma.

Honey's medicinal effect on human health can be achieved by either oral or topical use. Studies (Samarghandian et al., 2017) revealed that therapeutic benefits of honey when taken orally for the treatment of laryngitis, osteoporosis, gastrointestinal ulcers, anorexia, sleeplessness, and constipation, as well as hepatic, cardiovascular, and gastrointestinal issues. On the other hand, eczema, lip sores, sterile and infected wounds, vaginal lesions, burns, surgery scars, and athlete's foot are prescribed for topical application of honey [Ghashm et al., 2010]. Honey is an ancient therapy for the treatment of infected wounds that has just recently been 'rediscovered' by the medical profession, especially in cases when current therapeutic agents fail.

## Chemical composition

Although season, the environment, and the conditions during processing also play a role in the composition of honey, it mostly depends on the floral source.

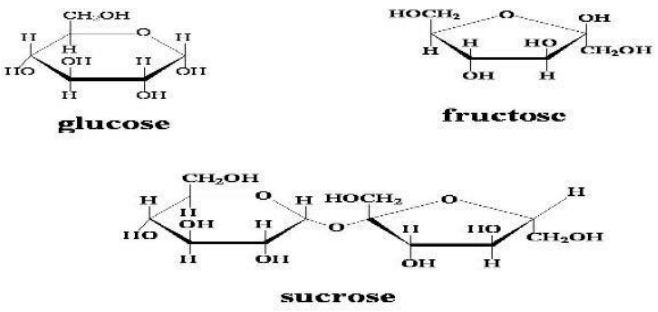



**Fig. 2.1**: Chemical composition of honey

## Carbohydrates

Three different species of sugar makeup the sugar in honey which is not a single species. These include common sugar (sucrose) which is in between 1 and 2 percent, and fruit sugar (fructose) which ranks the among the highest (41%) grape sugar, (glucose), which has about 34 percent (Cummings JH 2007). The ratio of one form of sugar to another is influenced by the source, such as floral pastures and to a lesser measure by the enzyme invertase, which dissolve normal sugar in grapes and other fruits. This enzyme can be found in both the flower where the bees get their nectarand inside the bees itself (Di Pasquale et al., 2013).

## Amino acids and proteins

Because nectar and pollen are essential components of plants, proteins can be found in honey. Proteins in honey can either take the form of simple chemicals like amino acids or more complicated structures (Alvarez-Suarez J 2013). Protein and amino acid content together make up no more than 0.7% of the total. Nearly all amino acids that are crucial for health are present in honey. Proline, the primary amino acid, is used to gauge how ripe honey is. Normal honeys should include more than 200 mg/kg of proline. Values below 180 mg/kg indicate that the honey has been likely been tampered with by the addition of sugar (Bogdanov et al., 2009).

## Aroma compounds and phenolics

The compounds behind honey's scent are called honey volatiles. Early in the 1960s, research on honey volatiles began. Recent research on volatiles recovered from honey (Bogdanov et al., 2002) revealed that while the majority of volatile chemicals are likely derived from plants, some are also likely to have been contributed by bees. Approximately 600 chemicals have so far been identified in various honeys. Secondary metabolites from plants include phenolic acids and polyphenols. These substances have been employed in plant systematics as chemotaxonomic markers. They have been proposed as potential indicators for identifying the botanical source of honey (Bogdanov et al., 2004). According to reports, light-colored honeys contain more flavonoids

and phenolic acid derivatives than dark-colored ones.

**HMF (HydroxyMethylFurfuraldehyde)**

According to (lichtenthaler et al., 2002), HMF is a six-carbon heterocyclic organic molecule with both aldehyde and alcohol (hydroxymethyl) functional groups. Furan moieties are the heart of the structure's ring, as opposed to the two functional groups. HMF is a solid, yellow material that is easily soluble in water but has a low melting point. HMF is a by-product of fructose's (one of honey's primary sugars) breakdown that develops gradually and naturally during honey storage but considerably more quickly when heating. The amount of HMF present in honey serves as a benchmark for the degree of heating; the higher the HMF number, the poorer the honey's quality is regarded as being (Tosi et al., 2004).

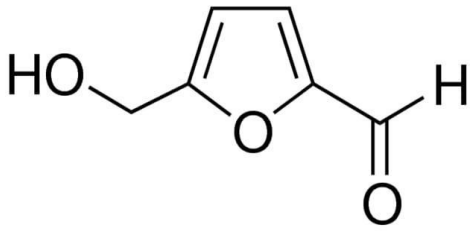



**Fig.2.2:** HydroxyMethylFurfuraldehyde

(Source:https://www.sigmaaldrich.com/IN/en/product/aldrich/w501808)

**Minerals and trace elements**

Mineral compounds are present in honey in various concentrations. In addition to several other components, potassium is the major element in honey. The main mineral element is potassium, which makes up an average of around one third of the total, however there are many different trace elements. Numerous studies have revealed that the trace element composition of honey is mostly influenced by its botanical source. 3.68 percent is what minerals have (Mattoon WR. et al.). Minerals in honey increase the value of honey for human consumption even if this portion of the honey is not produced in great quantities. The majority of the minerals are found in honey, including silicon, iron, manganese, calcium, sodium, phosphorus, chlorine, and sulphur (Aili et al., 2014). Darker honey varieties contain more minerals than lighter ones when

compared to the observed mean value. Of course, a darker speciescan be found that is less wealthy than certain lighter species.

**Acidity and pH Acids**

PH and acidic Honey contains acids as well. In the past, it was thought that bees would inject their venom into the honeycomb's cell walls along with the honey to preserve it. Formic acid is one of the primary components of bee venom, hence it was assumed that the honey also contains formic acid (Machado et al., 2018). Due to this, some people have even advised against using honey. According to studies, honey is primarily constituted of apple and lemon acids, which are entirely separate acids. As a buffer, honey prevents the pH from being altered by the addition of minor amounts of acids and bases. The presence of phosphates, carbonates, and other mineral salts contributes to the buffer's capacity (Molan et al., 2006).

**Water**

The quality factor that influences how long honey will stay fresh and resist bacterial fermentation is its water content (the amount of water in honey). Raw honey can contain less than 14 % water, and the higher the perceived value of the honey, the less water it contains (Hatjina et al., 2014). It is widely accepted that high-quality honey should be treated with a water concentration of less than 20%. Low water level is preferred since honey with a water content of more than 20% may start to ferment and lose its fresh quality. Wild yeast causes unpasteurized honey to ferment. Although these yeasts are less likely to produce fermentation in honey with low water content because of the high sugar concentration in honey (Maughan et al., 2002). One of the most popular natural sweeteners is honey. It can be described as a naturally occurring food that mostly consists of sugars and water, with modest amounts of additional nutrients like vitamins, minerals, amino acids, organic acids, flavonoids, and other phenolic compounds and aromatic molecules. Depending on the materials' botanical sources, its composition varies substantially.

**Global numbers for Honey economy and India’s market share**

According to the most recent figures, global output is expected to exceed 1.5

million tonnes per year, with a market value of around 4000 million US dollars. China is the world's top producer and exporter of honey, with over 300,000 tonnes valued at more than $250 million in 2013. According to production patterns, China dominates global honey sales. Mexico is the second-largest exporter in the world, after Germany (Faostat et al., 2016). With a (Compound annual growth) CAGR of around 4.8 percent, the natural honey industry is currently valued at US$ 8.4 billion and is predicted to reach US$ 10.3 billion by 2025. A total of 1,779.6 metric tonnes of honey are produced worldwide. China produces more than 28% of the honey consumed worldwide, followed by Turkey (5.9%), Iran (4.5%), and the United States (4%). India produces 3.5 percent of the world's honey, making it the sixth-largest producer in the world. India's honey exports increased from US$ 56.2 million to US$ 100.8 millionover the last decade, growing at a pace of 6.5 percent per year, outpacing global export growth.

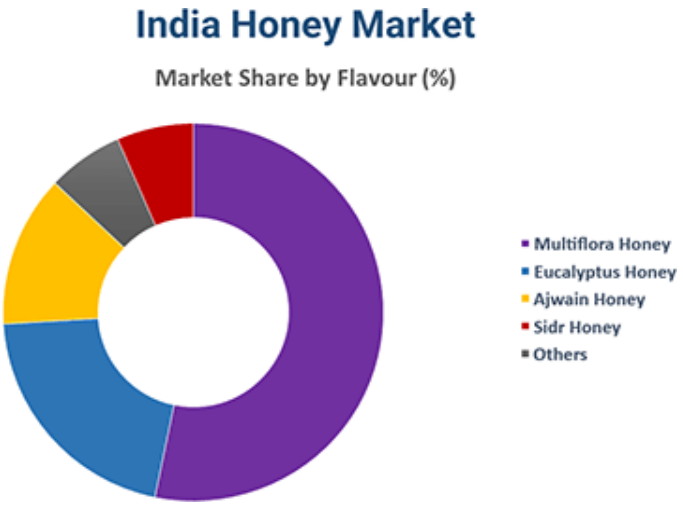



**Fig.2.3:** Market share by flavour ( source: https://www.expertmarketresearch.com/reports/india-honey-market)

Furthermore, with US$ 1.9 million in imports in 2019, India's imports are almost non- existent. India's honey is primarily exported to the United States, Saudi Arabia, and the United Arab Emirates. In 2020, the Indian honey market will be worth INR 17.29 billion, thanks to the growing popularity of online retail businesses. The industry is predicted to increase at a CAGR of 10% over the forecast period of 2022-2027, owing to rising therapeutic use of honey and its increasing relevance as a quality ingredient, particularly in nutraceuticals. By 2026, the industry is expected to be worth INR 30.6 million. Multiflora honey,

eucalyptus honey, ajwain honey, sidr honey, and other honeytypes can be found on the market. In India, multifloral honey accounts for the majority of total market share, which is extremely fragmented due to the vast number of manufacturers.

**Honey adulteration**

Adulteration is a severe problem in India that puts many people's health at danger. Adulterated food is dangerous since it can be toxic and affect one's health, as well as deprive a person of nutrients necessary for appropriate growth and development. Honey can be adulterated for a number of reasons, such as adding sugar to improve the flavour in response to consumer demand or increasing production by blending inexpensive, low-quality honey with more expensive honey. Low-cost sugars and store-bought syrups are regularly used in honey adulteration.

**Importance of adulteration**

An adulterant is a chemical which act as contaminant adulterants when combined with other substance. Adulterants are compound that are added to pure substance in order to increase the amount while lowering the quality for economic and technical benefits.

**Honey adulterants**

Corn syrup (CS), High fructose corn syrup HFCS, glucose syrup (GS), sucrose syrup (SS), inverted syrup (IS), and high fructose inulin syrup (HFIS) as well-known adulterants from sugar cane and sugar beet. Enzymatic activity, electrical conductivity, and the presence of certain compounds are only a few of the chemical and biological characteristics of honey that are altered by sugar adulteration (Soares et al., 2017).

**Cane sugar**

Two sugar molecules known as monosaccharides make up sucrose (glucose and fructose). The disaccharide sucrose is made up of the monosaccharides glucose and fructose, which share the same chemical formula ($C_6H_{12}O_6$) but have different chemical conformations ($C_{12}H_{22}O_{11}$). Sugar cane, a perennial C4 grass, is commonly used to extract juice, which is then purified chemically

and physically, the water is evaporated off, and the sugar crystals are separated (Ruiz-Matute et al., 2007). Plants that utilise the Hatch-Slack cycle (C4 metabolic pathway) produce cane sugar, whereas plants that utilise the C3 metabolic pathway create nectar (Calvin cycle).

**Corn syrup**

High fructose corn syrup (HFCS), often known as corn syrup, is a viscous liquid that is colourless, odourless, and has a viscosity greater than that of water. Corn syrup is a liquid sweetener used in cooking that is produced by hydrolysing corn starch. Based on the amount of fructose it contains, corn syrup is divided into three categories: HFCS-42 (42 percent fructose), HFCS-55 (55 percent fructose), and HFCS-90 (90 percent fructose). Fructose from high fructose corn syrup must be stored in the liveras fat or glycogen since it cannot be used to make energy. As a result, the body is unable to properly metabolise the extra fructose in HFCS (Olivares et al., 2012).

**Palm sugar**

Sugar from palm trees Palm sugar is made by extracting the flower buds of the palmtree. It's a chemical-free natural sweetener that goes through only a few stages. Sucrose was found to be the most abundant carbohydrate in palm sugar, followed by glucose and fructose, according to one study (Yamada T., 2015). Palm sugar has a big benefit over other sugars in that it does not cause blood sugar spikes due to its low glycemic index (35). In India, the most common honey adulterant is jaggery syrup, which is made from the evaporation of palm tree sap (Luis et al., 2012).

**Invert sugar**

Invert sugar (IS) is created when sucrose breaks down into its monosaccharides, fructose and dextrose. To execute the inversion method, heat the sucrose syrup in the presence of acids, alkalis, or invertases. IS mimics the sugar profile of pure honey by obtaining its sugar from beet and cane plants (Gehlawat et al., 2001). In the beverage and food industries, invert sugar is used to make non-crystallized milk,jams, fake honey, and liquid sugar (Se K.W et al., 2018). Due to the fact that beet is a C3 plant, inverted beet syrup is a well-

known adulterant that may be tailored to approximate the natural sucrose (glucose-fructose) composition of honey. In one study, samples of clover, orange, and buckwheat honey were each mixed with varying amounts of inverted beet syrup (Veana, 2018). Generally speaking, invert sugar is regarded as a medicine that is safe and has no known negative effects. Patients with diabetes mellitus, rare genetic illnesses of fructose intolerance, glucose-galactose malabsorption, or sucrase-isomaltase deficiency, and those who are sucrase-isomaltase deficient, should exercise caution (Paradkar et al., 2002).

**Rice syrup**

One of the most popular honey adulterants in China is rice syrup (RS), which is produced by hydrolyzing rice polysaccharides and is similar to beet syrup. Maltotriose (52%), maltose (45%), and glucose (5%) are the three sugars that make up rice syrup. Because maltose is made up of two molecules of glucose and maltotriose is made up of three, rice syrup behaves in the body like pure glucose. Recently, RS- contaminated honey has started to show up on the market. The Calvin cycle ofphotosynthesis in rice syrup, a C3 syrup adulterant, is similar to that of natural honey. As a result, the use of rice syrup as an adulterant in honey is a significant issue that affects quality assurance and food safety [Du et al., 2015].

**Insulin syrup**

Inulin is a naturally occurring polysaccharide that belongs to the fructans family. These dietary fibres are made up of a chain of fructose residues with glucose at the end. The fructan type is determined by the fructose molecules' bond patterns. The chain of 2-1 connected fructose in inulin, for example, has been terminated by glucose. Wheat, onion, bananas, garlic, asparagus, sunchoke, and chicory are all good sources of this polysaccharide. A nectar honey sample was also mixed with varied amounts of high fructose inulin syrup (5, 10, and 20 percent, weight for weight) to simulate honey adulteration (Siddiqui et al., 2017).

**Selection of adulterants**

Honey adulterates are chosen based on three factors: the geographical origin,

the financial advantages, and the accessibility of sugars or other sweeteners. One well- known instance of adulteration is the use of rice and wheat syrup extraction in Turkey and France (Soares et al., 2017). Heating vegetable juices or partial enzymatic hydrolysis can be used to extract plant syrups (Spiteri et al., 2016). European countries, according to (Corradini et al., 2012), adulterate honey with HFIS. Common sweeteners used in commercial honey adulteration will be explored in this section, as well as their health effects. Figure 4 depicted the chemical structures of common sugar adulterants in honey.

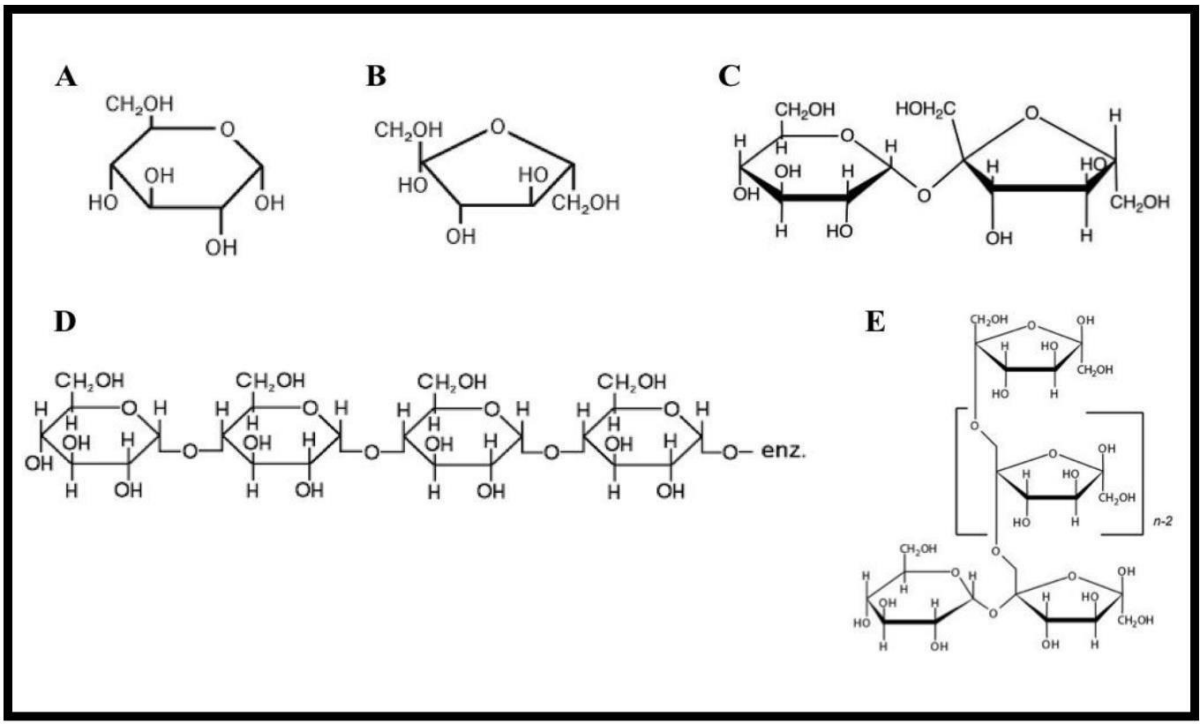



**Fig.2.4**: Chemical structures of common sugar adulterants in honey. (A) Glucose, (B) Fructose, (C) Sucrose, (D) Rice syrup, and (E) Insulin syrup. ( source: https://www.mdpi.com/2304-8158/9/11/1538/htm)

**Methods of adulteration**

Adulteration in commercial honey is usually classed as direct, indirect, or blending. Aspreviously mentioned, the authors of one article (Zabrodska et al., 2015) divided honey adulteration into two categories: direct and indirect.

**Direct Adulteration** direct addition sugar syrups is a post-production process for increasing honey sweetness by adding certain ratios.

**Indirect adulteration** happens when bees are overfed honey, pesticides, and industrial carbohydrates during the primary nectar period in order to retrieve more honey from colonies.

**Blending** is characterised as the blending of pure, premium honey with cheap, inferior honey. It has been difficult to detect adulterants and new method to distinguish pure and contaminated honey.

### Harmful effect of honey adulteration

Pure honey, also known as natural honey, is a pre-digested meal that can be used as a sweetener by many people who are unable to digest cane sugar. It could come from either nectar from flowers or plant sap digested by the bee's stomach (Codex 2001; Rahman et al. 2013). It contains 80 percent simple carbohydrates like fructose and glucose, which the digestive system can easily break down. As a result, it is absorbed directly into our bloodstream and turned into energy. Pure honey contains around 200 components, including water, vitamins, minerals, phenolic acids, proteins, and enzymes, in addition to simple sugar (Bogdanov et al., 2008).

### Various instrumentation

Infrared spectroscopy

The determination of the botanical origin can be done using several infrared absorption ranges. Seven unifloral and two multifloral honey species have each been authenticated using near-infrared spectroscopy (Ruoff et al., 2006)

### NEAR-INFRARED SPECTROSCOPY

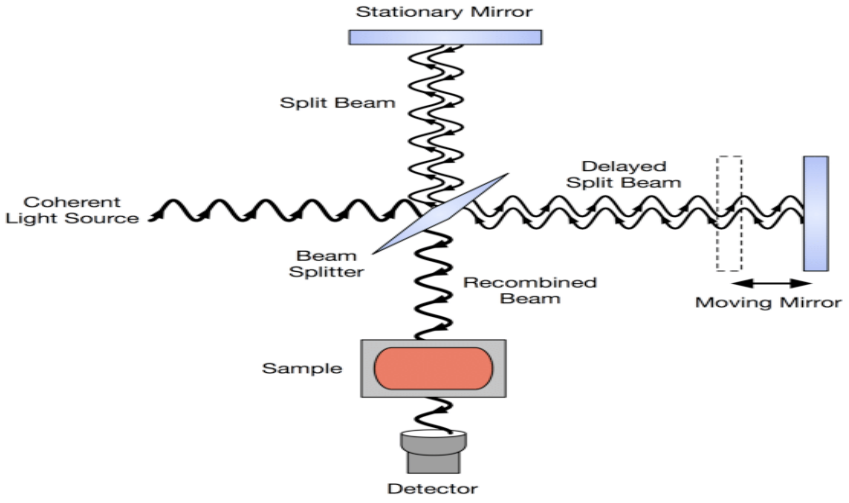



Fig.2.5: FT-NIR Diagram

(Source:https://www.researchgate.net/figure/Schematic-of-a-typical-FT-NIR-instrument)

### Working principles

NIR spectroscopy is a non-destructive analytical tool that has shown great potential for non-targeted analysis or fingerprinting of food samples. Near-infrared radiation falls in the 780-2500 nm (10000 – 4000 cm-1) range of the electromagnetic spectrum, which is composed of energy waves that can penetrate organic matter and excite the molecular bonds present (Abbas *et al.*, 2012; Bazar *et al.*, 2016). NIR spectroscopy is essentially the measurement of vibrational transitions that occur when molecular bonds, which have an energy gap of a specific magnitude between their ground and fundamental state, are excited with radiation. Intra- and intermolecular bonds become excited and enter this fundamental state due to energy of equivalent magnitude being absorbed from incident radiation (Manley, 2014). Bonds containing hydrogen almost always absorb within the NIR region, making this type of analysis appropriate for organic samples. Bond vibrations of -CH, -NH, -OH and -SH, which are prevalent in organic molecules, are observed in the 800-2500 nm region (Roggo *et al.*, 2007), while more specifically the region of interest for NIR food applications is 1100 – 2500 nm (Norris, 2009). An NIR spectrum is an average spectrum based on the excitation of the whole sample, making it best-suited to the analysis of homogenous samples, such as honey. Near-infrared region spectra typically contain broad absorption bands instead of sharp and resolved peaks seen originating from the MIR region of 2500-25000 nm. This is due to the excitation of various overtone bands in the NIR region corresponding to the fundamental vibrations found in the MIR region, which creates a spectrum of crowded and severely overlapping peaks, in contrast to the distinct shifts produced in the MIR region (Cozzolino *et al.*; 2011; Abbas *et al.*; 2012; Manley, 2014).

## GAS CHROMATOGRAPHY-MASS SPECTROMETRYWORKING PRINCIPLE:

The molecular identification and separation of chemical mixtures are accomplished by the GC/MS apparatus (the MS component). It is among the most accurate pieces of equipment available for analysing environmental samples. The GC operates by heating a mixture, which causes it to split into several components. The sample is placed in the GC's inlet where it is vaporised by the carrier gas (helium) and sucked into a chromatographic column. The chemical components of the mixture of interest are separated as the sample is passed through the column by their interactions with the stationary phase of the column's coating and the carrier gas (mobile phase). The final part of the column terminates at the intake of the ion source, where compounds eluting from the column are converted to ions, after passing via a heated transfer line. The sample molecules are ionised by an electron stream, which creates molecular ions and smaller ions with differing relative abundances that act as a "fingerprint" for that particular chemical structure. The mass analyzer separates the ions before they are detected.

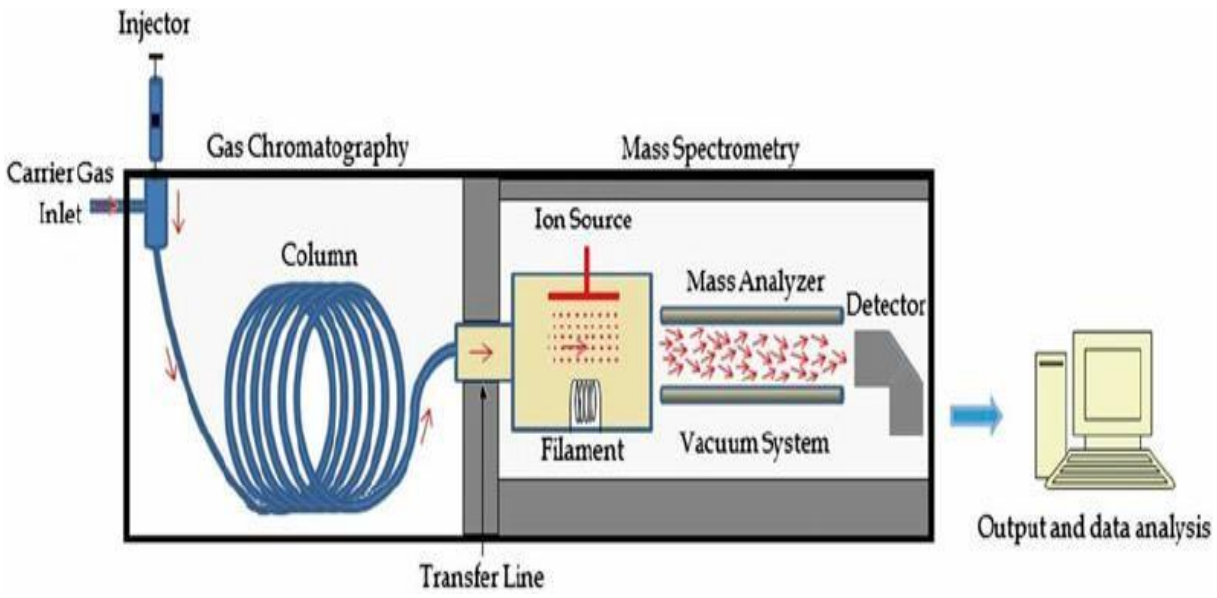



**Fig.2.6:** GC- MS Diagram

(Source:https://www.researchgate.net/figure/Schematic-plot-of- the-main-components-of-GC-MS-instruments)

# MATERIALS AND METHODS

# 4. MATERIALS AND METHODS

**Collection of Sample material:**

For this study, a total of 455 honey sample were examined. 315 monofloral honey samples Taramira (*Eruca vesicaria*), Neem (*Azadiracta indica*), Coriander (*Coriandrum sativum*), Jamun (*Syzygium cumini*), Sidr (*Ziziphus spina-christi*), Ajwain (*Tracyspermum ammi*), Eucalyptus (*Eucalyptus globulus*), were collected. The two remaining multifloral honey were obtained from beekeepers, Uttar Pradesh. All the samples were kept at room temperature for further analysis.

**Honey spiking**

Each of the authentic honey samples were spiked with one form of sugar (glucose syrup) in ratio: 60/40.

**Reagents and Chemicals:**

- Glucose
- Fructose
- Ethanol (HPLC grade)
- Methoxyamine Hydrochloride
- Pyridine
- N, O-Bis(trimethylsilyl) trifluoroacetamide (MSTFA)

**INSTRUMENTS:**

- Weighing Balance
- Vortex
- Sonicator
- Centrifuge
- Lyophilizer
- Thermo mixer

## Methodology

### FT-NIR spectroscopy:

FT-NIR spectra was recorded using a Thermo Scientific Antaris II FT-NIR Spectrophotometer in the transflectance mode was used to collect the near-infrared (NIR) spectra. NIR spectra were acquired with a resolution of 4 cm-1 and 64 scans spanning a spectral range of 4,000–10,000 cm-1. Figure shows a typical FT-NIR spectrum of honey about 3g of honey was poured into a transparent zip bags and to covered the transflection plate. Each sample contains two replicates were averaged to provide a single average spectrum.

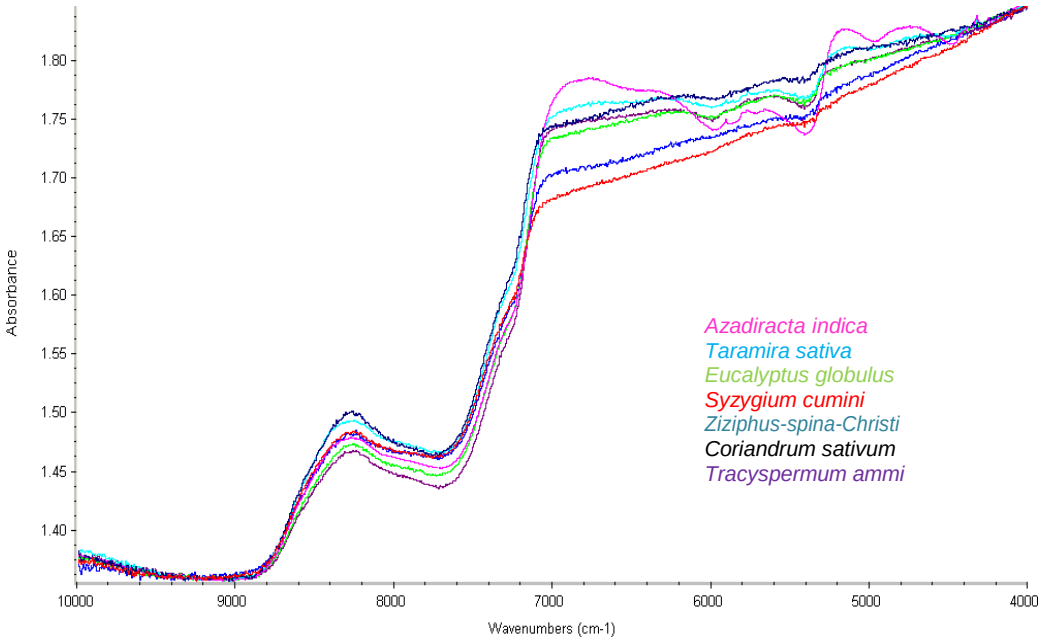



**Figure No.3.1**: FT-NIR spectra of seven unifloral honey types

### DD-SIMCA Model

Soft independent modelling of class analogy (SIMCA) is a class modelling method or one-class classifier commonly used in chemometrics for authentication purpose. The original version of SIMCA, proposed by S. Wold has numerous modifications mostly related to the way of constructing the acceptance boundaries. A well-known modification is DD-SIMCA. A straight forward but effective one-class classification method, SIMCA (Soft Independent Modelling of Class Analogy) is mostly based on Principal component analysis

(PCA). The main idea is to exclusively use samples and objects from one class to build a PCA model, and then categorise new objects depending on how well the model fits them.

The classification of DD-SIMCA model using different spectra to authenticate honey samples. Comparatively, it is observed that DD-SIMCA always obtained better results in terms of sensitivity and specificity in both the training and test sets. The DD- SIMCA models constructed with the training samples and test samples presented, respectively. There is no extreme objects identified in the models in the training set, respectively. All the training samples that fall within the boundary delimited by the established green line considering an α-value error of 0.01 are depicted in green circles, while extreme objects are illustrated in orange circles between the green and red lines, and outliers in red circles outside the red line, respectively, as can be seen in the Acceptance plots. These findings can be confirmed in their respective Extreme plots constructed using the training samples. Therefore, only the DD-SIMCA models constructed for the authentication purposes because their posteriori sensitivities (100% respectively) the predefined α-value of 0.01. In other words, the cut-off level is calculated assuming that of the training objects are expected to be extremes. Comparing the predictive ability of the best models constructed using the test samples, the DD-SIMCA model misclassified seven authentic honey and seven adulterated samples while the DD-SIMCA model correctly classified all the samples achieving a 100% of sensitivity and specificity.

**Extract sample preparation for GC-MS:**

- 100mg honey sample taken in 1.5ml eppendorf.
- Add 1ml 100% ethanol in each eppendorf
- Vortex for 10 minutes at 1800 rpm.
- Sonicated the sample for 5 minutes at room temperature.
- Vortex the sample for 1 minutes then centrifuge for 10 minutes at 1200 rpm.
- Collected the supernatant in new eppendorf.
- Lyophilised the sample and reconstitute the honey sample.

**Derivatization of the honey sample**

- Weighed 20mg Methoxy amine hydrochloride in a microcentrifuge tube.
- Add 1ml pyridine.
- Vortex for 3 minutes for proper mixing.
- The final concentration of Methoxyamine hydrochloride in pyridine is 20mg/ml.
- Taken lyophilized sample in a microcentrifuge tube and add 60µL Methoxy aminehydrochloride solution.
- Vigrously vortex mixed for 1 minute.
- Kept at 65˚C for 30 minute.
- Add 90µL of MSTFA (with1% TMCS).
- Vortex for mixing properly.
- Keep at 70˚C for 60 minutes.
- Centrifuge for 5 minutes at 12000rpm at room temperature.
- Taken supernatant and transfer it into the glass vial.
- Inject in GC-MS for analysis.

**GC-MS analysis of honey samples:**

GC-MS analysis of Ethanol solvent of honey was performed using the equipment Perkin Elmer Clarius 680 gas chromatography with the SQ 8 mass selective detector. The equipment has an Elite 5 MS with dimension of 30 m×0.25µm film. The equipment has an Elite 5 MS with a dimension of 30 m × 250 µm having film thicknesses of 0.25 µm was used for obtaining the peak separation in the chromatogram. Helium in a split ratio of 3:1 and a flow rate of 1.5ml/min was used as the carrier gas. The running condition for the samples was 70℃ for 2 min as initial hold and heating ramp of 12.5 °C/min until the temperature reaches 295°C and finally, with a ramp rate of 25°C/min temperature reaches 320℃ with 3 min as a final hold. Mass spectrometry was conducted at 250 °C as a transfer line and ion source temperature while, 70 eV ionization potential, and 50 to 565 atomic mass units scan range and solvent delay was 5.50 minutes. The carrier gas was helium with a flow rate of 1ml min. Injection volume was 0.5µL and split ratio was 500:1.

# RESULTS AND DISCUSSION

# 5. RESULTS AND DISCUSSION

## NIR combined with DD-SIMCA classifies honey samples specific to medicinalplants

The classification method Soft Independent Modelling of Class Analogies (SIMCA) is such a method that each class of samples is described by its own principal component model. Thus, in principle, any degree of data co-linearity can be accommodated by the models. This method is mainly based on PCA. To create a PCA model using only samples/object belonging to a class and classify new objects based on how good model can fit them. PCA component analysis was used for data reduction.

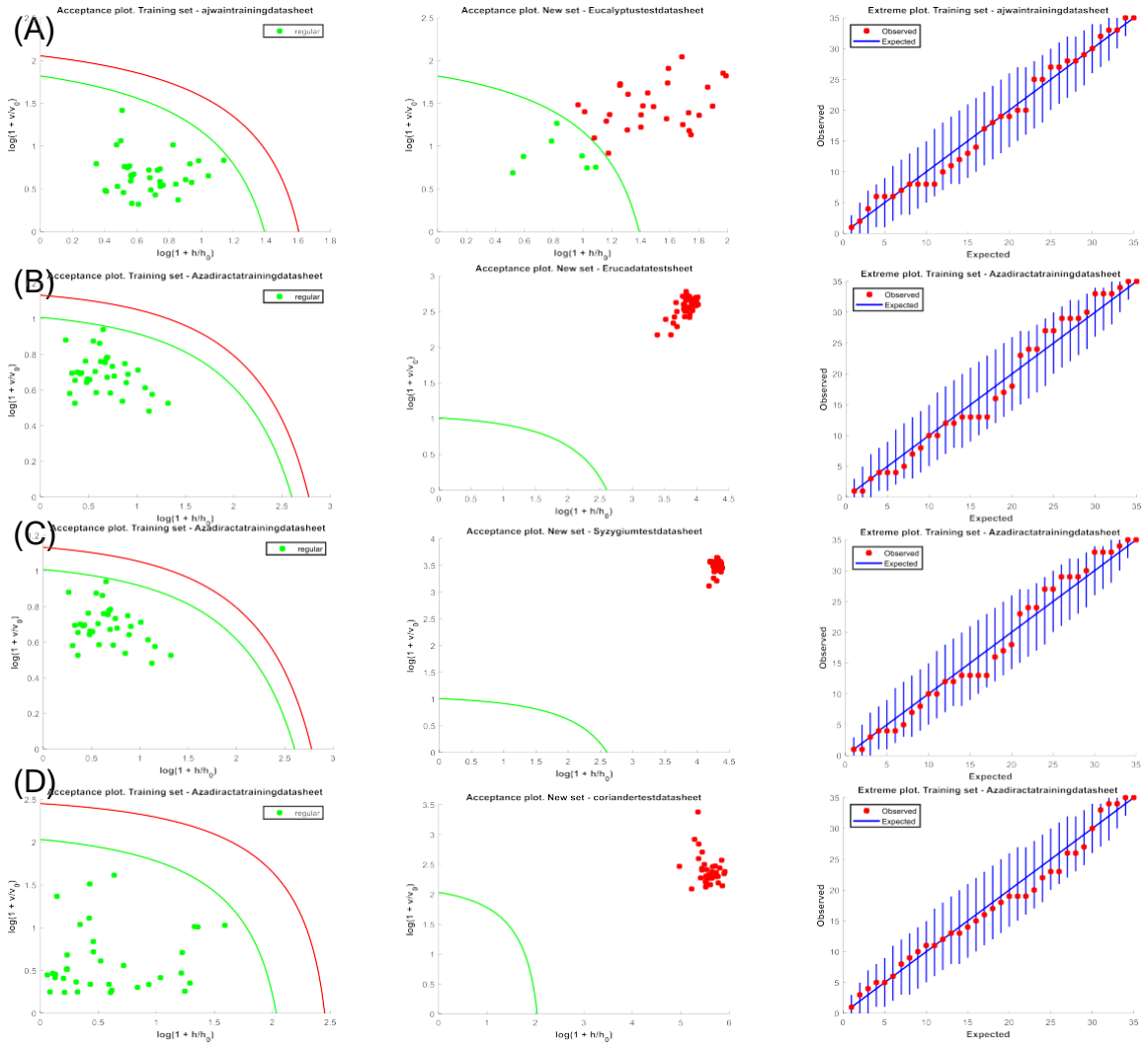


**Figure No. 4.1:** The application of DD-SIMCA for the classification of unifloral honey samples. A) *Trachyspermum ammi* honey (n=35) vs *Eucalyptus globulus honey* (n=35); B) *Azadiracta indica* honey (n=35) vs Eruca sativa honey (n=35); C) *Azadiracta indica* honey (n=35) vs *Syzygium cumini* honey (n=35); D) *Azadiracta indica* honey (n=35) vs *Coriandrum sativum* honey (n=35). The acceptance plot for training set provides a graphic representation of the acceptance area, the area inside the green curve with the threshold for α = 0.01.

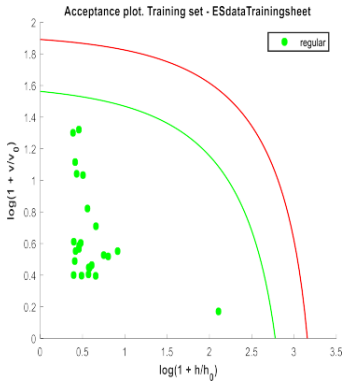
**TABLE 1 Shows DD-SIMCA plots of one type of vs another type of honey**

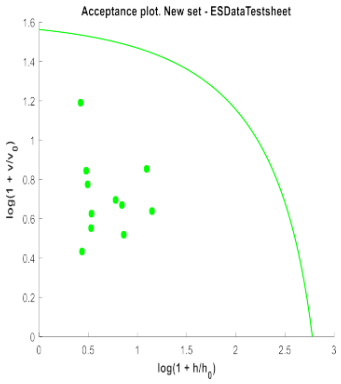
| Types | Training set | Test set | PC | $\alpha$ | $\beta$ | DOF (SD) | DOF (OD) | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|---|---|
| *Azadiracta indica* (Neem) *Eruca sativa* (Taramira) | 35 | 35 | 5 | 0.010000 | 0 | 6 | 43 | 100 | 100 |
| *Azadiracta indica* (Neem) *Syzygium cumini* (Jamun) | 35 | 35 | 5 | 0.010000 | 0 | 6 | 43 | 100 | 100 |
| *Azadiracta indica* (Neem) *Coriandrum* sativum (Coriander) | 35 | 35 | 2 | 0.010000 | 0 | 2 | 2 | 100 | 100 |
| *Tracyspermum ammi* (Ajwain) *Eucalyptus globulus* (Eucalyptus) | 35 | 35 | 5 | 0.010000 | 0.45698 | 12 | 7 | 100 | 100 |

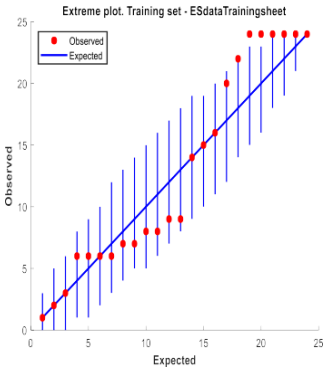
Table 1 shows the DD-SIMCA models constructed in which 35 training set and 35 test take to make the DD-SIMCA Plots for the authentication purposes. For making better

plots change the principal component till 5 only because their posteriori sensitivities (100%) the predefined α-value of 0.01 and the β-value is different in most of the samples. Comparing the predictive ability of the best models constructed using the test samples, the DD-SIMCA model misclassified seven authentic honey while the DD SIMCA model correctly classified all the samples achieving a 100% of sensitivity and 100% specificity.

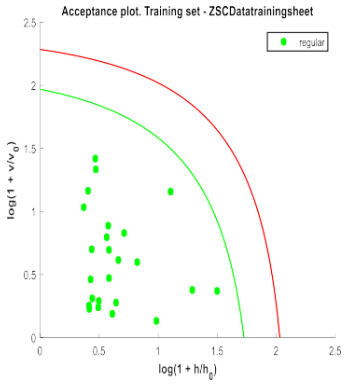
(A)

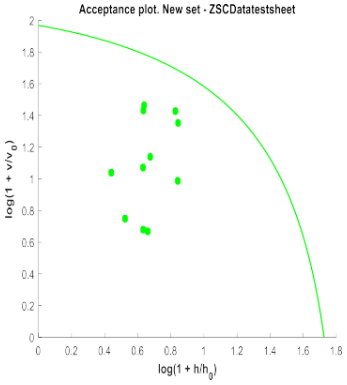


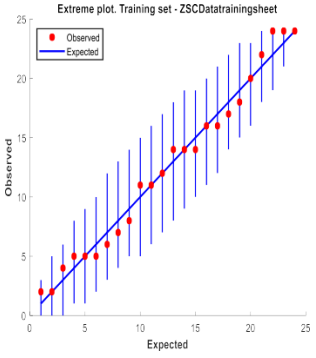



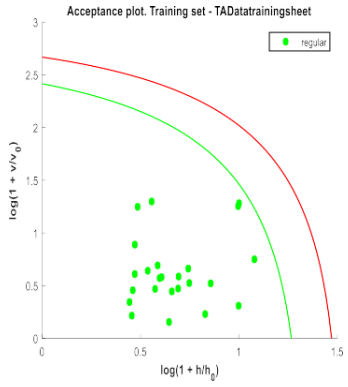

(B)

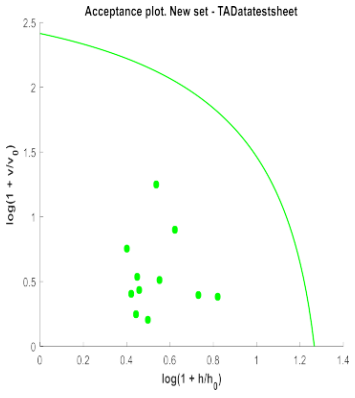


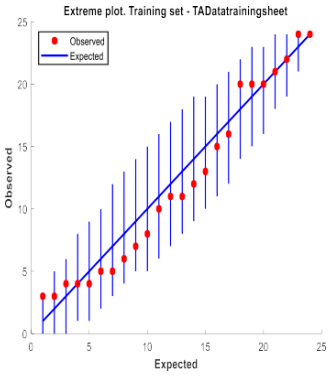




(C)



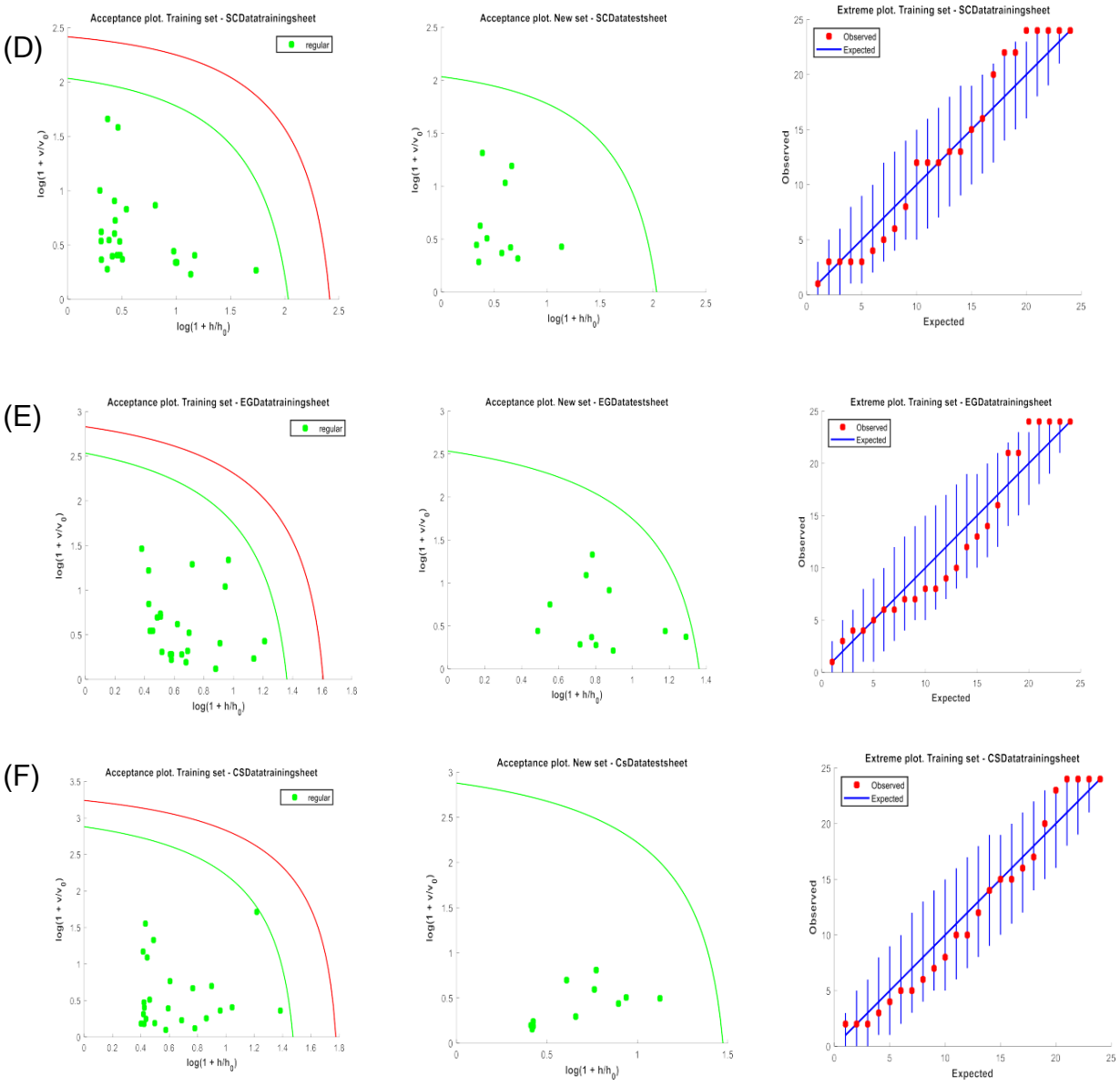



**Figure No. 4.2:** The application of DD-SIMCA for the classification of unifloral honey samples. A) *Eruca sativa* honey (n=24) vs *Eruca sativa* honey (n=11); B) *Ziziphus-spina christi* honey (n=24) vs Ziziphus-*spina christi* honey (n=11); C) *Trachyspermum ammi* honey (n=24) vs *Trachyspermum ammi* honey (n=11); D) *Syzygium cumini* honey (n=24) vs Syzygium cumini honey (n=11); E) Eucalyptus globulus honey (n=24) vs Eucalyptus globulus honey (n=11); F) Coriandrum sativum honey (n=24) vs Coriandrum sativum honey (n=11); G) Azadiracta indica honey (n=24) vs Azadiracta indica honey (n=11); The acceptance plot for training set provides a graphic representation of the acceptance area, the area inside the green curve with the threshold for α = 0.01.

## TABLE 2 OF DD-SIMCA PLOTS

| Types | Training set | Test set | PC | $\alpha$ | $\beta$ | DOF (SD) | DOF (OD) | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|---|---|
| *Eruca sativa* (Taramira) | 24 | 11 | 2 | 0.010000 | 0.99993 | 1 | 4 | 100 | 100 |
| *Zizyphus-spina-christi* (sidr) | 24 | 11 | 2 | 0.010000 | 0.98757 | 4 | 3 | 100 | 100 |
| *Trachyspermum ammi* (Ajwain) | 24 | 11 | 2 | 0.010000 | 0.99997 | 12 | 3 | 100 | 100 |
| *Syzygium cumini* (Jamun) | 24 | 11 | 3 | 0.010000 | 0.99983 | 2 | 2 | 100 | 100 |
| *Eucalyptus globulus* (Eucalyptus) | 24 | 11 | | 0.010000 | 0.97437 | 8 | 2 | 100 | 100 |
| *Coriandrum sativum* (Coriander) | 24 | 11 | 2 | 0.010000 | 0.9983 | 5 | 1 | 100 | 100 |
| *Azadiracta indica* (Neem) | 24 | 11 | 3 | 0.010000 | 0.98249 | 5 | 3 | 100 | 100 |

Table 2 shows the classification of DD-SIMCA model using different spectra to authenticate honey samples. Comparatively, it is observed that DD-SIMCA always obtained better results in terms of sensitivity and specificity in both the training and test sets. DD SIMCA model is one class classification using this model classify the botanical origin of honey. The DD SIMCA models were constructed for honey varieties Taramira (*Eruca vesicaria*), Neem (*Azadiracta indica*), Coriander (*Coriandrum sativum*), Jamun (*Syzygium cumini*), Sidr (*Ziziphus spina-christi*), Ajwain (*Tracyspermum ammi*), Eucalyptus (*Eucalyptus globulus*) in all the samples screen 70 % as a training set and 30% as a test set. After performing the DD SIMCA none of the samples were identified no extreme objects in the models of the training set. All the training samples that fall within the boundary delimited by the established green line considering α-value error of

0.01 are depicted in green circles, while extreme objects are illustrated in orange circles between the green and red lines, and outliers in red circles outside the red line as can be seen in the acceptance plots. These findings can be confirmed in their respective Extreme plots constructed using the training samples. Therefore, only the DD-SIMCA models constructed for the authentication purposes because their posteriori sensitivities (100%) the predefined α-value of 0.01 and the β-value is different in most of the samples. Comparing the predictive ability of the best models constructed using the test samples, the DD-SIMCA model misclassified seven authentic honey while the DD SIMCA model correctly classified all the samples achieving a 100% of sensitivity and 100% specificity.

## GC-MS ANALYSIS

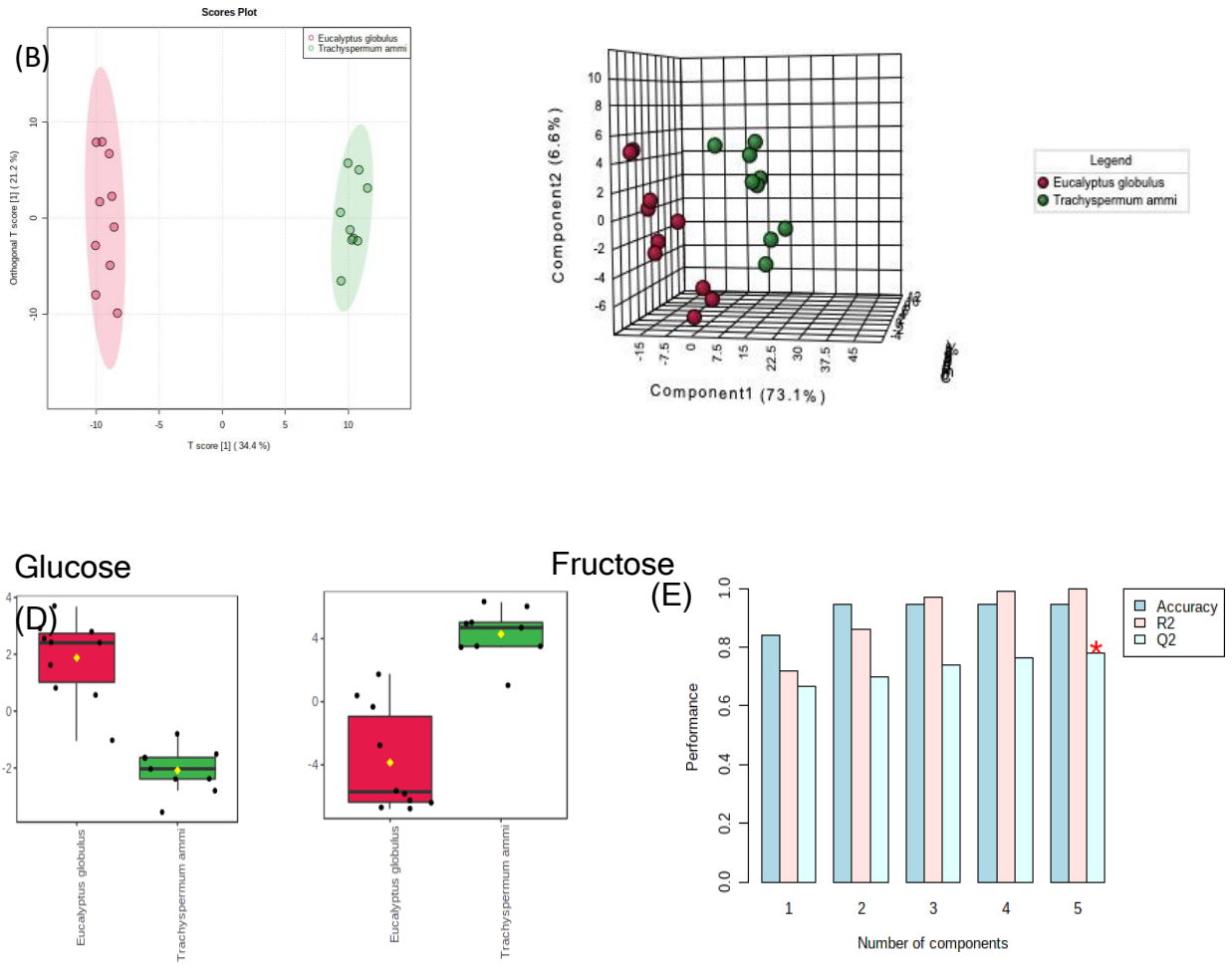



**Figure No. 4.3:** Differentiation of honey samples. A) Honey samples of *Eucalyptus globulus* and *Tracyspermum ammi*. B) Score plot (2D) obtained from the Principal component analysis. C) Box plot for VOC Compound identifying the difference of Glucose and Fructose sugar of *Eucalyptus globulus* and *Tracyspermum ammi*. D) Internal cross validation graph

The raw GC-MS files were converted into net CDF format. These net CDF files were imported to R based script for automated peak detection, retention time alignment and peak matching. Software was used for data processing. The data matrix containing (m/z), samples and intensities for further was used for statistical analysis. Multivariate statistical analysis was performed. Principal component analysis (PCA) was performed in order to have a better visualization of all the information contained in the data set. It is possible to visualize the

differences among the various groups by projecting the object of the data set into principal component. Overall, 75 samples including the 10 samples of *Eruca sativa*, 10 samples of *Azadiracta indica*, 10 samples of *Coriandrum sativum*, 10 samples of *Syzygium cumini*, 10 samples of *Ziziphus-spina-christi*, 10 samples of *Trachyspermum ammi*, 10 samples of *Eucalyptus globulus* were analysed. PCA classification identifying a clusters between these two groups one is Eucalyptus globuls and the other one is Tracyspermum ammi (as shown in figure 9). In 3D structure of score plot of honey (as shown in fig 9) principal component 1 (73.1%) and the principal component 2 (6.6%) value. And this classification is based on two metabolites one is glucose and the other one is fructose. And these are further validating internal cross validation in this the number of principal component is 5.

# CONCLUSION

# CONCLUSION

Honey have various medical purposes, including boosting serotonin, antioxidants, and immunity. It also have antimicrobial properties, lowers stress and anxiety, and have many other benefits. In present study, I explored NIR combined with chemo metrics models to successfully classify the botanical origin of honey. The offset correction produced the best results for DD-SIMCA, achieving 100% sensitivity and specificity in the test set and 100% sensitivity in the training set. The suggested methodology therefore utilised as a quick and effective instrument to authenticate honey and stop its adulteration.

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