1. INTRODUCTION

*Other bees, like soldiers, armed in their stings,*

*Make boot upon the summer’s velvet buds,*

*Which pillage they with merry march bring home.*

*Shakespeare, King Henry*

Honey bees are social insects that belong to the genus *Apis*. They are primarily distinguished by the production and storage of honey and the construction of colonial nests out of wax. The hive is like a giant nursery, home and factory where the queen, workers and drones lives in colonial harmony. The beehive comprises a densely packed group of hexagonal prismatic cells made of beeswax, called a honeycomb. The bees use the cells to store food (honey and pollen) and to house the brood (eggs, larvae and pupae) (Fig.1.1).

![Honey bee hive with stored honey, pollen and brood.](image)

**Fig.1.1:** Honey bee hive with stored honey, pollen and brood.

**Queen** is the only fertile female in the colony. She lives for about 3-5 years, has a longer abdomen as compared to drones and workers and is capable of laying 2000-2500 eggs per day. Queen larvae are fed exclusively on royal jelly.

**Drones** are the unfertilized males having bigger eyes and their sole job is to mate with the queen at about 2-8 weeks of age. After mating with the queen the drone dies.
**Introduction**

**Workers**: are sterile females constituting the largest population of the hive and responsible for caring for young, food storage, cleaning, foraging, protection, tending of the queen, regulating the temperature of the hive and construction of comb. They have a pollen basket on their hind leg for collecting pollen and four pairs of special glands on the underside of their abdomen to secrete bees wax. The ovipositor is modified into a sting with a poison gland that secretes venom and serves for defense.

Honey bees are master chemists and chemical engineers. The success of bees in the animal kingdom is largely because of the chemistry and the uses of their products: honey, beeswax, bee venom, propolis, pollen and royal jelly. Three of these products (venom, royal jelly and beeswax) are chemically synthesized by the bees themselves. The other three (pollen, propolis and honey) are derived from plants and are modified and engineered by the bees for their own use.

All these products have amazing benefits. The bees and the entire honey bee colony could not survive but for the various uses to which their products are put. The benefits of these products to man are as yet underused, understated and sometimes even misunderstood.

**Honey Bee Products**

Honey bees are regarded as big money makers for the agriculturist; bees collect various substances from the plants, add their own secretions to them, process them in the hive and finally allow them to ripen. These ripened substances serve as commercial bee hive products.

---

**Fig.1.2: Honey bee products**
Honey: Honey is an aromatic, sweet, viscous liquid produced by honey bees from the nectar of plants and is economically the most important as well as the most well known product of the bee hive.

The chemical composition of honey is very complex. It mainly contains sugars along with a small quantity of acids, minerals, vitamins, enzymes and antibiotic substances. The sugars chiefly comprise two reducing sugars *i.e.* dextrose and laevulose (fructose), which upon human consumption pass from the stomach to the surface of the mucous membrane, where they are absorbed directly into blood. Honey is hygroscopic which means, it absorbs moisture from air. Honey, also known as **liquid gold** is a product incomparable to anything else in terms of nourishment and medicinal properties. Its regular use is recommended in case of severe malnutrition. In human nutrition, it is specifically useful for growing children and is used mostly by those who are engaged in hard labor and by athletes, who require instant source of energy. Interestingly it is reported that the first man who climbed Mount Everest, Edmund Hillary, “boosted up” his strength with a few tea-spoons of honey. Kumar *et al.* (2008) reported that honey has wound healing properties and by strengthening the immune cells it helps to fight microbial diseases.

Bee pollen: Also known as “The Life Giving Dust”. Bee pollen is a fine powder like material collected by honey bees from the anthers or the male gametophyte of flowering plants. Worker bees have pollen baskets on their hind legs which facilitate pollen transportation to the hive. Bees roll the pollen grains into pollen balls with the aid of their salivary secretion, these are called pollen pellets and these pollen pellets are then pushed in to the corbicula or “Pollen Basket”.

In the hive, pollen pellets are stored in pollen cells. Bee pollen is low in calories but rich in proteins, amino acids, vitamins, minerals, enzymes, beneficial fatty acids, carbohydrates and phytochemicals such as flavonoids, carotenoids and phytosteroids (Broadhurst, 1999). It helps in reviving damaged cells, accelerates recuperation after influenza, allays nervousness and strengthens physical resistance.

Bee bread: It is modified pollen, processed and stored by bees in the pollen cells of the comb. For this, bees knead the pollen collected and stored in the pollen cells with honey and saliva and process this mixture for fermentation. Saliva contains
different enzymes like amylase and catalase, and with the help of these enzymes coupled with microorganisms, moisture and temperature (35-36 °C) of the bee hive, bee pollen gets ripened into bee bread in about two weeks. Bee bread differs from the freshly collected bee pollen. It is the raw material for production of bee milk and royal jelly, which the young nurse bees make with the help of secretions from glands in their heads.

Due to fermentation its stabilization increases and its digestibility and nutritive value also increases. This is consumed by the developing bees (Herbert and Shimanuki, 1978).

Bee bread has a different composition than pollen. Bee bread contains fewer proteins than the original pollen, but they are easier to absorb. The moisture content decreases considerably to 13 or 14% through drying after the harvest. Bee bread also contains the following substances: proteins with essential amino acid, vitamins C, B1, B2, E, H (biotin), K, P (rutin), nicotonic acid, folic acid and B5 (pantothenic acid), pigments, carotenoids and anthocyanins, the enzymes saccharase, amylase and phosphatase, flavonoids, more than 25 different minerals and spore elements such as iron, calcium, magnesium, phosphorus, potassium, copper, zinc and selenium.

Since the amount of lactic acid in bee bread is about six times greater than in pollen, it has a higher acidity and thus a lower pH value. This acidity of bee bread makes it self-preserving: it inhibits the growth of moulds and other micro-organisms so bee bread does not become mouldy as quickly as pollen.

Propolis: It is a resinous mixture that honey bees collect from flower buds, sap flows or other botanical sources.

It is excreted as drops from the tree's bark if it is cut or cracked. The bees bring it on their hind legs, just like pollen, to the hive. They mix it with their own wax and saliva resulting in the formation of propolis, which is used as a sealant for unwanted open spaces, protection of the brood in the hive and also provides protection from the attack of microorganisms.

The chemical composition of propolis is extremely complex. It contains more than 150 components such as flavonoids, phenolic acids and their esters, ketones, alcohols, amino acids and inorganic compounds (Hagazi et al., 2000; Banskota et al.,
Introduction

2001; Marcucci et al., 2001; Bankova, 2005). Propolis has been reported to possesses antioxidative, antiulcer, antimicrobial and antitumor activities (Lotfy, 2006).

Beeswax: This organic substance is secreted by four pairs of glands on the underside of the abdomen of worker honey bees 10-12 days of age. While producing and building the comb with wax, bees eat and digest a lot of honey owing to their high metabolic requirements. This leads to a high body and surrounding temperature, which is necessary to keep the wax fluid. The wax drips like a curtain out of the gland’s narrow opening and hardens as it comes in contact with the air. It then changes into transparent, white, ellipsoidal scales. The bees hold these scales with their forelegs and chew them into the right form to build a comb.

Pure bee’s wax is white, but when it is mixed with other substances such as pollen, propolis etc it becomes yellow. It can be extracted from cappings of sealed honey cells and by rendering old, distorted or damaged combs.

It is produced by bees to make their wax nest and is an important commercial product. Today it finds a large market in the cosmetic industry, Catholic church candles and in commercial polishes and medicines (Kreill, 1996)

Royal jelly: It is the queen bee’s principle food and is produced by the mandibular and hypopharyngeal glands of nurse bees. The young bees add secretions from these glands present in their heads, to the ingested bee bread to make bee milk or royal jelly. They put this bee milk in cells that contain young larvae. The larvae of worker bees, drones and queen eat this product for the first three days of their life which helps in their growth. Subsequently, only the queen larvae and the adult queen are fed on this material others being fed bee bread.

The bee milk is made up of two components i.e. a clear and a milky white fluid. Royal jelly consists of approximately equal parts of these two fluids, whereas the bee milk for the drones and workers is mostly made up of the clear component. Worker bees produce maximum bee milk when they are a week old; after three weeks, the secretion stops the duty of the worker changes and they begin to go outside to collect nectar and pollen.

Royal jelly contains a high concentration of vitamins B5, B6, and amino acids and has a special rejuvenating substance that promotes tissue growth, muscle and cell regeneration. It also has anti-apoptotic, antioxidant and free radical scavenging activity (Karadeniz et al., 2011).
**Bee venom:** Worker bees and the queen, have an extendable stinger at the end of their abdomen. The queen usually uses this to lay eggs, but she can also sting with it when required. The worker bees do not lay eggs normally and use the sting for defense. This sting is left in the body of the victim as a consequence of which the worker bee dies.

A drop of fluid *i.e.* the bee venom, sits on the extended stinger. Bee venom is synthesized in the venom glands of bees and is stored in the venom sac at the base of the stinger (Fig 1.3). Young bees have little venom. Their venom sac is not filled until the 15th to 20th day of life, when it contains about 0.3 mg of liquid venom. The spring bees that are raised with a lot of pollen have the maximum and most effective venom. Bee venom dissolves best in water.

![Honey bee sting](image)

**Fig.1.3: Honey bee sting**

The production of venom increases during the first two weeks of the adult worker’s life and attains a maximum level when the worker bee becomes involved in foraging and hive defense. It is a unique weapon possessed by the bees and has a prime role to play in defense of the bee colony.

Bee venom is a complex mixture containing simple organic molecules, proteins, peptides, phospholipids, physiologically active amines, amino acids, sugars, volatile pheromones, minerals and other bioactive elements. Kreill (1996) reported that mellitin was the most abundant active component present in bee venom which is antimicrobial, anti-inflammatory and antiviral in nature.
Introduction

Toxicity of the compounds present in bee venom has been known to man since ages. These components are responsible for many toxic or allergic reactions in different organisms, such as local pain, itching, irritation, moderate or severe allergic reactions and from mild inflammation to death.

In Chinese medicine, bee venom has long been used for the treatment of rheumatoid arthritis. It has also been found to have antibacterial activity against Gram positive and Gram negative bacteria with Gram positive bacteria being more sensitive.

Oxidative stress:

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidant molecules and enzymes. Free radical is an oxygen containing molecule that has one or more unpaired electrons, making it highly reactive with other molecules. Oxygen by-products are relatively unreactive but some of these can undergo metabolism within the biological system to give rise to these highly reactive oxidants. Not all reactive oxygen species are harmful to the body. Some of them are useful in killing invading pathogens or microbes. Reactive oxygen species are capable of disrupting nearly any metabolic pathway through their attack on proteins, lipids, and nucleic acids (Hybertson et al., 2011).

Overproduction of free radicals has been related to nutritional, environmental and microbial stress conditions. Nutritional stress including the hyper secretion of Vitamin A, deficiencies of some vitamins like Vitamin E, some minerals like Mn, Zn and Se. Iron overload, high levels of poly unsaturated fatty acids, presence of different toxins and consumption of toxic compounds leads to generation of free radicals. Environmental stress including the radiations, increased temperature and humidity is reported to be related to overproduction of free radicals. McDowell. (2007) also reported oxidative stress due to bacterial, viral and fungal diseases.

Free radicals can chemically interact with cell components such as DNA, proteins or lipids and steal their electrons in order to become stabilized. This, in turn, destabilizes the cell component molecules which then seek and steal an electron from another molecule, therefore triggering a large chain of free radical reactions. Much of the damage is caused by hydroxyl radicals generated from \( \text{H}_2\text{O}_2 \) via the Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents (possibly NADH) to regenerate the metal. Lipids are major targets during oxidative stress. Free radicals can attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane bound proteins significantly. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins (Humpries and Sweda, 1998)

Source: Japan Institute for the control of ageing, NIKKEN SEIL Co., Ltd
Unlike reactive free radicals, aldehydes are rather long lived and can therefore, diffuse from the site of their origin and reach and attack targets which are distant from the initial free-radical event, acting as “second toxic messengers” of the complex chain reactions initiated. Among the different aldehydes which can form during lipid peroxidation, the most intensively studied are malondialdehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (HNE) (Esterbauer et al., 1991)


DNA is also a vulnerable target for free radical attack where they can attack both the base and the sugar moieties of polynucleotide are prone to attack thus producing single and double strand breaks in the DNA, producing the adducts of bases and sugars and also leads to cross-links to other molecules, by which lesions are produced (Sies and Menck, 1992; Sies, 1993)

Aerobic organisms use molecular oxygen (O₂) for respiration or oxidation of nutrients to obtain energy. Reactive by-products of oxygen, such as superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂) and the highly reactive hydroxyl radicals (·OH), are generated continuously in cells grown aerobically. Most of such products are derived from sequential univalent reductions of molecular oxygen catalyzed by several membrane associated respiratory chain enzymes. Experimental data indicate that in *Escherichia coli*, the respiratory chain can account for as much as 87% of the total H₂O₂ production (González-Flecha and Demple, 1995).
Environmental agents including ionizing radiations, UV radiations or numerous compounds that generate intracellular O$_2^-$ (redox-cycling agents such as menadione and paraquat) can cause oxidative stress, which arises when the concentration of active oxygen increases to a level that exceeds the cell’s defense capacity. Some immune cells which use NADPH oxidase enzyme, upon invasion by pathogenic bacteria, also exploit oxidative stress as a weapon during phagocytosis.

*Staphylococcus aureus* is an important cause of oxidative stress (Teixeira et al., 2008; Chakraborty et al., 2011). As is known phagocytosis is the major mechanism of defense against extracellular microbes. *S. aureus* is able to survive within phagocytic cells both in polymorphonuclear leukocytes (PMN) and monocytes. Hence it is very difficult to deal with staphylococcal infections.

**Damage caused by oxidative stress:** Oxidative stress leads to many pathophysiologi cal conditions in the body. Some of these include neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, gene mutations and cancers, chronic fatigue syndrome, fragile X syndrome, heart and blood vessel disorders, atherosclerosis, heart failure, heart attack and inflammatory diseases.
Fig. 1.4 Clipping from newspaper showing skin disease due to oxidative stress (Times of India)

Fig. 1.5 Effect of oxidative stress on cells

To counter this, living organisms have to build up mechanisms to protect themselves against oxidative stress, with enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione (Cabisco et al. 2000).

**Antioxidants:** Every cell that utilizes enzymes and oxygen to perform functions is exposed to oxygen free radical reactions that have the potential to cause serious damage to the cell. Antioxidants are molecules present in cells that prevent these reactions by donating an electron to the free radicals without becoming destabilized themselves. Free radicals are normally neutralized by efficient systems in the body that include the antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and the nutrient-derived antioxidant small molecules (vitamin E, vitamin C, carotenes, flavonoids, glutathione, uric acid and taurine) (Nagai et al., 2001).
In healthy individuals, a delicate balance exists between free radicals and antioxidants. In some pathologic conditions such as diabetes, and in critically ill patients, oxidative stress causes the level of antioxidants to fall below normal. Antioxidant supplements for such conditions are expected to be of benefit. As a preventive measure against certain diseases, the best approach for healthy individuals is to regularly consume adequate amounts of antioxidant rich foods, e.g. fruits and vegetables. According to Desai et al. (2010) antioxidants work by two methods: first those which inhibit the production of free radicals and are called the preventive antioxidants e.g. catalase and the second are called the chain breakers which interrupt especially the propagation phase e.g. vitamin E and SOD.

In the present study various honey bee products will be screened for their in vitro activity against a range of organisms. Further propolis will be evaluated for antimicrobial and antioxidative potential in staphylococci induced systemic infection in mice. The proposed studies will contribute to the identification of natural products obtained from honey bees, having therapeutic efficacy against microorganisms.

**Staphylococcus aureus:**

*Staphylococcus* also known as Golden staph and Oro staphira is a member of the Firmicutes. It is characterized by the following features: Gram-positive, non-motile, non spore forming, asexually reproducing (binary fission), and round-shaped
bacterium. It is frequently found in the nose, respiratory tract and on the skin. It is a facultative anaerobe positive for catalase and nitrate reduction. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Stefani *et al.* (2012) reported that MRSA could be hospital/health care acquired (HA); community acquired (CA) or could also be livestock associated.

**Fig. 1.6: Drug resistance in *S. aureus***

*S. aureus* is responsible for causing chronic/relapsing diseases and is reported to persist as an opportunistic intracellular organism both under *in vitro* and *in vivo* conditions (Brouillette *et al.*, 2003). *S. aureus* has developed resistance to most classes of antimicrobial agents. Chakraborty *et al.* (2011) evaluated the possible protective effects of nanoconjugated vancomycin against vancomycin resistant *S. aureus* (VRSA) infection on selective markers of oxidative damage and antioxidant status in liver and kidney. Despite much research and development there is no approved vaccine for *S. aureus*
**Aim and Objectives:**

The potential therapeutic application of bee products which is giving rise to an alternative healing system referred to as Apitherapy provided the incentive for the design of the present study. In the present study, the antimicrobial property of bee products including pollen, propolis, honey and venom has been evaluated. Further the therapeutic potential of propolis has been studied and compared with antibiotics ampicillin and amoxicillin against *S. aureus*. The following objectives were covered under the investigations:

1) **In vitro** evaluation of honey bee products such as pollen, propolis, honey and bee venom for activity against following types of organisms:

   **a) Bacteria: Both Gram positive and Gram negative organisms were used viz.**
   
   - *E. coli*
   - *Bacillus subtilis*
   - *Staphylococcus*
   - *Salmonella*
   - *Streptococcus*
   - *Pseudomonas*

   **b) Fungi:**
   
   - *Saccharomyces cerevisiae*
   - *Candida albicans*

   **c) Helminths: (Amphistomes)**
   
   - *Gastrothylax crumenifer* (Creplin, 1847) Poirier, 1983

2) **In vivo** studies to evaluate the therapeutic potential of propolis (antioxidative and tissue protective) alone or in combination with standard antibiotic against *Staphylococcus* infected BALB/c mice.