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Biochemical and Biological Evaluation of Propolis

by

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Dedicated to ALLAH Almighty, Hazrat Muhammad (PBUH) and my brother Mohsin Raza who has been a constant source of motivation and encouragement during the challenges and supporting me spiritually throughout my life.

CERTIFICATE OF APPROVAL

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Abstract

It has been announced that more than 1/2 of the pharmaceutical products currently used in clinical use are derived from natural products. Natural products or materials extracted as potential drugs from natural resources have been reported to be safer, with zero or minimal toxicity. At present, there is growing hobby in Propolis treatments because of the consequences related to the artificial drug remedy and Propolis is one in all such natural substance with the drug capacity. Currently, there is a growing hobby in Propolis treatments due to the effects of the artificial drug remedy and Propolis is one with the drug capacity in all such natural substance. The selected samples of Propolis extract: Propolis 1, Propolis 2, Propolis 3, Propolis 4, were collected from four different locations. These Propolis extracts were screened for antioxidant, an tibacterial, antifungal, cytototoxicity, while FT-IR analysis was used for qualitative analysis. the extraction technique was Manual maceration. The results of DPPH assay revealed that noteworthy percentage of free radical scavenging was higher observed in Propolis 2 than Propolis 1, 3, 4 with the value of 84 ± 0.1 and IC50 value is 45.0 and Propolis 1, value of 45 ± 0.1 and IC50 value is 19.0 and the value of Propolis 3, 61 ± 0.1 and IC50 value is 33. And Propolis 4, value of 55 ± 0.1 and IC50 value is 28 at 30 concentrations respectively and % scavenging of Propolis 2 in term of IC50 and P-value is < 0.001 was higher significance than Propolis 1, 3, 4. On the contrary, Propolis 1, 3, 4 extract showed less cytotoxicity, antioxidant and antifungal potential. All of the extract of strains was found to have significant antifungal activity, the maximum percentage of zone of inhibition of fungal strains of Propolis 2, 2 is higher than Propolis 1, 3, 4 i.e *Fusarium solani* was 79 ± 0.1mm and and Propolis 1, was 43.3 ± 0.1mm and Propolis 3, was 62.2 ± 0.1mm Propolis 4, was 51 ± 0.1mm respectively. The Minimum percentage of zone of inhibition of Propolis 1and Propolis 2 i.e. *Aspergilus niger* was 29 ± 0.01mm and 16 ± 0.01mm Propolis 3 and Propolis 4, 21 ± 0.01mm, 19 ± 0.01mm respectively, the assay was run as triplicate analysis. All of the two extracts of Propolis have antibacterial activity against Five bacterial strains tested, most active being the Propolis 1 showed maximum activity against *Staphylococcus aureus* (0.4 ± 0.1mm) and *Salmonella arunes* (0.3

± 0.1mm) (MIC <100). The weakest activity of Propolis 1 was observed against E.coli. i.e. 0.019 ± 0.01mm respectively. Propolis 2 showed maximum activity against E.coli (0.1 ± 0.4 mm) and *Staphylococcus aureus* (0.2 ± 0.8mm) (MIC <100). The weakest activity of Propolis 2 was observed against *B. subtilis* (0.21 ± 0.1). Propolis 3 showed maximum activity against. *Staphylococcus aureus* (0.5 ± 0.1mm) and *E. aerogenes* (0.3 ± 0.1mm) (MIC < 100). The weakest activity of Propolis 3 was observed against *Salmonella arunes* i.e. 0.017 ± 0.01mm respectively. Propolis 4 showed maximum activity against *salmonella arunes* (0.6 ± 0.1 mm) and B. *subtilis* (0.4 ± 0.1mm) (MIC < 100). The weakest activity of Propolis 4 was observed against *Staphylococcus aureus*(0.22 ± 0.6). In this research, three different concentration (1000ppm, 500ppm, 250ppm) of Propolis extract were used to test their toxic effect by using brine shrimps cytotoxic assays. The results are shown that Propolis 2, has maximum cytotoxicity and significant with percentage mortality of 98.66 ± 0.01 IC50 value of 230 μg/ml and p-value is < 0.001, followed by Propolis 1 with percentage mortality of 53.66 ± 0.01, IC50 value of 128 μg/ml and p-value is < 0.001, followed by Propolis 3 with percentage mortality of 77.66 ± 0.01, IC50 value of 180 μg/ml and p-value is < 0.001, followed by Propolis 4 with percentage mortality of 61.66 ± 0.01, IC50 value of 145 μg/ml and p-value is < 0.001, at 300 μg/ml concentration. The present study of tested Propolis extracts confirmed the presence of functional groups identified by an analysis of FT-IR spectroscopy were significant against the Carbonyl group (C = O). Our study investigated the natural ethno medicinally meaningful properties of Pakistan's variety of locally available Propolis, phytochemical evaluation of extracts with the evidence of their active phytochemical constituent that could be used effectively for natural treatment. The results showed that this Propolis extracts can be used safely in pharmaceuticals and other industries.

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Abbreviations

%FRSA	Percent Free Radical Scavenging Activity
AAE DMSO	Ascorbic Acid Equivalent
DPPH	2,2-Diphennyi-picry-Hydrazyl-Hydrate
FAO	Food and Organization
FT-IR	Fourier Transform Infrared spectroscopy
IC50	Median Inhibitory Concentration
LC50	Median Lethal Concentration
MIC	Minimum Inhibitory concentration
NA	Nutrient Agar
NB	Nutrient Agar
P1, P2, P3, P4	Propolis 1, Propolis2, Propolis 3, Propolis 4.
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
TAC	Total Antioxidant Capacity
ZOI	Zone of Inhibition

Chapter 1

Introduction

1.1 Background

Propolis is dark colored sticky materials collected from honeybees in their hives from plants. It is also recognized as bee gum. Honey bees use this material along with wax in construction of their nests [1]. Propolis was first introduced by authors in Ancient Greece as Pro means for Infront of e.g. entrance to and polis means city or community hence Propolis means any substance that is used in defense of the hive [2]. Bees use Propolis and apply it as thin layer on internal walls of their hives in their nests. Propolis also use for the repair and strengthening of the combs, and to make entrance of their hives weather tight or easier to defend [3].

Propolis has some mechanical and biological properties. Propolis contains substances that is responsible for putrefaction of bacteria and molds within the hive [4]. This property of Propolis is an essential characteristic due to which Propolis is also known as as medicinal agent. Propolis also possess, antifungal, antiviral, and antibacterial characteristics and also as antiulcer, local anesthetic, hepatoprotective, antitumor and immunostimulant etc [5]. These characteristics made Propolis a good substance to be used in medicine [6], as constituent in biocosmetics, health foods and many other purposes [7]. To handle and control diseases, current research has been focused for the usage of old natural medicinal product

to handle and control diseases. Resistance has caused increasing nosocomial infections in pathogen. Propolis is one of natural products that have been verified on pathogens and in other organisms that causing community acquired infections. As a well known pathogen, confrontation has also been seemed in opportunistic microorganisms [8].

In various forms of topical, Propolis is used as a natural remedy in various health food stores. It is also utilized in beauty products or as a prevalent alternative drug for self medication of different syndromes [8-9]. Recent uses of Propolis incorporate details are cold disorder (upper respiratory tract infection, influenza and common cold) and in addition to dermatological properties used in wound heal up, treatment of burns, genitalis, acne, neurodermatitis and herpes simplex [10].

Propolis became registered as an authorized drug in London. Between seventeenth and twentieth century in Europe, Propolis was very famous in Europe due to its antibacterial property. Glue bee is used as a violin varnish in Italy [11]. It was extensively used because of its heal up property in the end of the nineteenth century and due to decline observations of appetite recovery and lung problems and in several clinics for tuberculosis was employed in the Second World War. For the treatment of the wounds, burns [12-13].

It is utilized in toothpastes and mouth freshener and to treat gum disease and stomach. It is broadly utilized in human being nourishments and drinks. It is easily accessible in market as a cream, container, throat capsules, mouth wash arrangements and powder, furthermore in several filtered items through which the wax were extracted. Due to it is antioxidant, antiviral and antimicrobial characteristics, its broadly utilized in human being, animal's medication and pharmaceutical [14-15].

In earliest times temples kept bees and use honey for the making of medicines [16]. According to Vegia silva Bee honey used in embalming process and for conservation purpose. Propolis and it extracts are known to have positive effect on tissue regeneration for a long time ago [17].

Scheller introduced the term ethanol extracts of Propolis (EEP). Series of experiments in 1970 resulted in identification of 19 elements with use of EEP. It was also found that EEP helps the healing processes in impaired cartilage [18] as well as enhances the ossification in artificially induced bone defects [19-21]. Propolis was also useful to inhibit the growth, adherence and promote detachment of trophozoites[22]. Propolis revealed a cure rate between 56% to 65% whereas drug showed 40% treatment rate against Giardisais. Propolis was found to be effective against gastric ulceration by showing anti *H. pylori*, anti acidic, anti inflammatory and antihistaminergic activities [23].

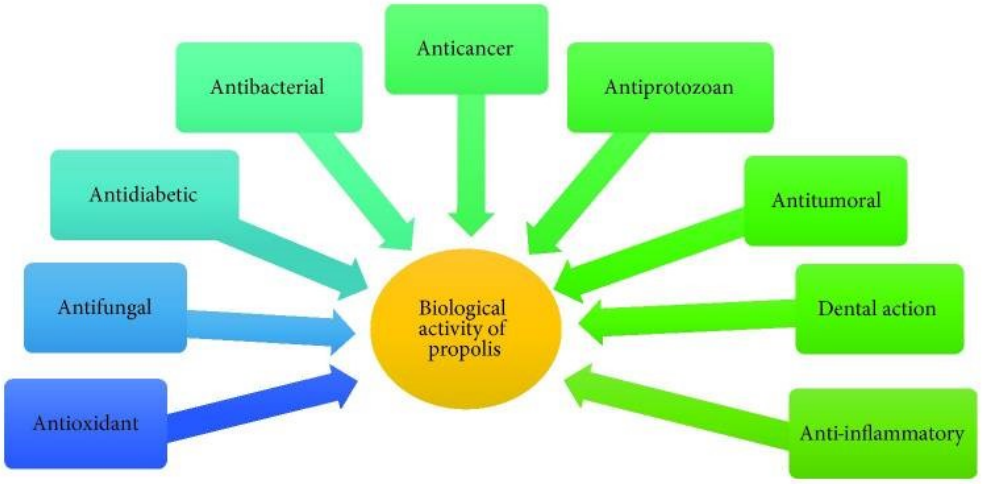

FIGURE 1.1: Bioactivity of Propolis

Propolis has wound healing capacity due to flavonoids, phenolic compounds, terpenes and enzymes that provide Propolis antifungal and antibacterial properties. It is also effective against the activity of free radicals in wound bed that make it favorable for repair process. [24]. Propolis is highly utilized in medicinal field that demonstrates the requirement of high quality and standardized Propolis preparations. For high quality Propolis gas chromatography, mass spectrometry and high performance liquid chromatography (HPLC) has been used for the analysis of Propolis extracts but analysis is difficult due to long analysis time and depends on many flavonoids and reagents that are expensive [25,26]. Propolis has outstanding therapeutic properties along with other honeybee products like (honey, royal jelly, pollen [27-30].

1.2 Problem Statement

Increasing attention is being given to natural resources in current era because of the problems of drug resistance, side effects associated with currently used drugs and the realization that nature provides a vast resource of active compounds with anti inflammatory, antimicrobial and anticancer therapeutic attributes. Putative and potential attributes have been described to Propolis in traditional medicines and a growing database on variety of diseases. There are approximately 300 phytochemicals that are identified in Propolis so far. The biological activities of Propolis vary significantly in chemical structure and subsequently, on the biotic activity, gathered at several times and from various phytogeographical areas. Therefore, it is recommended to analyze the Propolis of different botanical origin composed of variable biologically active substances with promising biological activities. [31,32]

1.3 Objectives

This study entails the following objectives:

- Collection of selected Propolis from different local areas of Pakistan.
- To investigate the biological activity using antimicrobial and antifungal assays
- To explore the natural ethno medicinally significant properties of variety of locally available Propolis of Pakistan.
- To perform the biochemical characterization of Propolis using FTIR

Chapter 2

Literature Review

2.1 Historical Perspective

Propolis is as ancient as a honey; also, it has been in use for a very long time for different purposes. There are records proposing the utilization of it by Egyptians, Persians, also Romans [33]. Old Egyptians delineated propolis-production honey bees on vases and also utilized it to treat the numerous sicknesses [34-35]. In the major century, Cornelius Celsius explained propolis as a treatment for treating injuries, and also for cure of boils [36]. Central Easterners has mentioned propolis also. For instance, Avicenna explained two several kinds of beeswax, that is, perfect beeswax also dark beeswax. He reported speaks by its solid smell it makes you wheeze also it has the attributes toward disposing of the spikes of the jolts also the stakes [37-39].

2.1.1 Propolis in Ancient Era

In past, propolis is used in conventional drug. Solely rare documents about use of propolis are available. Some sources as of the twelfth century define pharmaceutical measures using bee glue that was used for treatment of oral and pharyngeal infections and also for dental caries. Karabadini in his book Book of Medical

Treatment 1486 proposed that propolis is effective against dental deterioration [40-42]. Advantageously, the consciousness of therapeutic properties of propolis made in conventional society medication and, in addition, propolis was still widely utilized in home grown prescription on the regions of Eastern Europe. Therefore, propolis is also called Russian penicillin [43].

2.1.2 Propolis in Initial Modern Era

In Europe propolis was introduced through Renaissance theory that attracted the interest of people working in medicine field. The History of Plants (1597), makes the utilization of the organic compound or substances of poplar tree for curing purpose [44]. In Seventeenth century, the propolis has been included as an ingredient of drugs for healing purpose in England [45-47]. Nicolas Louis Vauquelin a chemist in 19th century emphasized propolis as a drug in the report of Society of Farming Vauquelin [48]. In that report he described the propolis as bee mastic that is collected by bees is resinous, yielding, odorant matter of reddish-brown color [49-50].

2.2 Propolis Bioactive Composition, Properties and Basis

2.2.1 Bioactive Proportion

Propolis is a compound combination made by bee honey discharge and plant-material derived mixtures. In more than three hundred elements were notable in several trials and new ones are quiet being well known throughout the chemical classification of novel type. Proportion of different elements exist in propolis, it relies on accumulation of time period and place. As it might be normal, unstable compounds (delivered by the source plants) are available in low quantity [55]. During the elaborations of propolis of bees over the resin's sugars are sup posed

to be introduced. Some composites are basic in very propolis trials and that one shows attributed properties. Various origin propolis comprises of various elements [56]. Because of various climatic condition, its biological activity is fluctuating in distinctive topographical origin trails [57]. For biological activity, the basic primary elements responsible are; fragrant acids, diterpenic acids, and polyphenols, yet not many diverse propolis forms have remained distinctive in principle elements of bioactive. Distinct arrangement is identified with flora particular region and managements of crude material [58].

2.2.2 Properties

When heated the propolis, it become soft, gummy, paliable and very sticky. It's a lipophilic in nature, brittle and hard material [51]. It has a specific and pleasing aromatic smell and differs in color from yellow green to red and to dark brown depending on its age and source [52, 53]. Even transparent propolis has been reported, depends on the resins of origin and it also ranges from yellow dark brown [54].

2.2.3 Liquefying Degree

Its delicate, stretchy and adhesive material at 25℃ -45℃. In solid state, it goes out to be very rigid and delicate. Even at high temperature, it will stay delicate after such usage. Over 45℃ it will turn out to be progressively sticky and gluey. Propolis will close to fluid in between 60℃ to 70℃ however in few examples; liquefying point might be high up to 100℃.

2.3 Solvency

Thinking about the arrangement of propolis, it can't utilize straight forwardly. Propolis exists separated commercially through appropriate solvent. Chloroform,

dichloromethane, ethanol, $(CH_3)_2CO$, water, ether, and methanol are the best widely utilized extraction solvents. A significant number of the bactericidal segments are dissolvable in H_2O liquor [59] which must expel all latent solid andreserve the requires mixture. Its synthesis relies on the geographic district and second one the technique for extraction, the dissolvable must be wisely selected [60].

2.4 Physical Properties of Propolis

Propolis has varies color due to different area and different source of plant. It is commercially extracted using suitable solvents such as ethanol methanol, chloroform, ether and acetone, but the best is ethanol) [61-62]. Propolis sample's biological activity varies because of its diverse geographical source. The estimated collection of the colony per annum is 150, 200 gram Propolis composition. Propolis is a complex resinous mixture that contains about 55% of resin and balsam, 40% of wax, 8% of essential and aromatic oils, 5% of pollen and 5% of impurities [63-65].

The color of Propolis varies from yellow to green,, green to red, and dark brown due to different source of plants. Almost 600 constituents have been recorded in propolis due to different sources and countries [66]. Many types of flavoindes like, ketones, waxyacids, aromatic aldehydes and alcohols, proteins, fatty acids, waxy acids, amino acids, steroids, sugars, vitamins, (B1, B3, B6, B5, B4, C, E) [67].

2.5 Phenols and Flavonoids

Within plant kingdom, The most boardly distributed groups of subtances is phenol is also called polyphenol [68-70]. Its most characteristic feature is its associated alcohol group (-OH) and aromatic ring. Phenol are further divided into, chromones, xanthones, stilbenes, anthraquinones, flavonoids, lignans, lignins and condensed tannins: simple phenols, benzoquinones, phenolic acids, phenylacetic

acids, hydroxycinnamic acids, phenylpropenes, coumarins [71-72]. The isolated phenol belong to different compound classes including ligans, Flavonoids, various acids. Until 2012, total 340 phenols were isolated in propolis and also 95 flavonoids and new phenols were isolated from propolis between 2013 and 2018. Flavonoids such as terpens ligans, and different derivatives of cinnamis acid and caffeoylquinic acids are also the main components of propolis. In Tropoical and Mediterranean regions, the active components of propolis are phenols [73-75]. Phenols are considered an important constituents of propolis, due to their abundance and activity [76]. At least 40 known propolis flavonoids were reported in 1995, whereas a minimum of 44 were also reported. Between 1995 and 2000, eight newly isolated flavonoids in propolis but only between 2000 and 2012 an astonishing 113 new Flavonoid isolated. Despite the high numbers of flavonoids already isolated, 92 (including their glycosides) were first discovered in propolis between 2013 and 2018 [77].

2.6 Terpenoids

The most diverse group of metabolites are terpenoids and terpenes. The term terpenes refers to hydrocarbon of molecules. Terpenoids are divided into sven class, monoterpenes, diterpenes, triterpenes, polyterpenes, uiterpenes, are Largest and most important compound group and most abundant volatile propolis component [78]. The propolis is the main biological and impotant subtances and also play a major role in determining its quality as wel. Basically, Terpenes were found in tropical propolis. The main groups of terpenes are, describe earlier, but they are all compounds play abetter role in pharmalogically [79].

2.7 Fatty Acids

Propolis have different parts, but one of non polar partis wax, or fatty acid. The first researcher was Heinen and Linskerns, isolate fatty acid from propolis. The

propolis contains many different types of fatty acid;saturated, monosaturated, polysaturated, omega 5, omega 6 [80].

2.8 Alcohols

Alcohols, sugar alcohols, setrols, and different types of alcoholic compounds including simple alcohol, fatty alcohols are all compounds present in propolis. Two new alcohols from propolis samples from Africa and Oman were isolate between 2013 and 2018 [81-82].

2.9 Antimicrobial Properties of Propolis

Antibectrial activity of propolis linked to the direct action on microorganisim. The analysis of propolis mechanisms allows to inhibited the effect on the permeability of the microorganism cell membrane and distruption of membrane potential and production of ATP [83]. It observed that the antimicrobial effect of propolis is higher in Gram negetive becteria as compare to Gram postive becteria because the main reson is the presence of outer membrane and production of hydrolysis enzyme [84-85] The ethanolic extract of propolis containg different compound like, pcoumaric panin artepillin C showed antioxidant as wel as antibacterial effect against *S. aurores* [86].

Cheliea et al, describe the antibacterial effect of propolis againts different becteia like S. aurens, *E. coli.* the activity of polyphenols and different compounds like epigenins and waxy and different acids shows highest role to inihibited the growth of becteria [88-87]. Different compounds, apigenin acts against Gram negitive becteria like *E. coli*, *S. entrica, E. aeregenis*, because apigenin with B-lactam antibiotics play a major role to resist the becteria.

Cinnamic acid is the aromatic group found in green plants and different parts like flower. Propolis is a rich source of cinnamic acid and esters as a material.

The antibectrial effect of propolis against different becteria like, *Aeromonas spp. Vibrio spp. E. coli, L. monocytogenes, Mycobacterium tuberculosis, Bacillus spp. Staphylococcus spp. Streptococcus pyogenes, serotype Typhimurium, Enterobacter-cloacae* [88, 89]. Cinnamic acid and its derivatives inhibit bacteria by spoiling the cell membrane, inhibiting the formation of ATPases, cell division [90-93].

The lipozyme reaction increases the activity of antimicrobials against *Staphylococcus epidermidis* and *Propionibacterium acne* [94]. The assessment of the antibacterial activity of propolis extracts has recently been based on the determination of total phenolics content (TP) andflavonoids (FP). Bridi et al. found in their study that TP and FP tests are not always adequately reflective of in vitro antimicrobial activity [95, 96].

The TP results were directly proportional to the flavonoid content and antioxidant properties in the samples with the highest and lowest content. In the case of antibacterial activity, they were however not unambiguos. It is suggested that other tests e.g. ORAC (Oxygen Radical Absorbance Capacity) and antimicrobial tests, should be considered in setting international quality standards for propolis [97-100].

2.10 Antifungal Action

With the presence of flavonoids, the fungicidal influence is associated [101-104]. And also influence of propolis on juice fungi spoilage *C. glabrata, Pichiaohmeri, C. kefyr, C. parapsilosis, C. pelliculosa, Candidafamata.* Within the 40 centuries of strains of *C. glabrata, C. krusei, C. albicans* and *Trichosporon spp* the propolis is a honey product with greatest antifungal action as verified [105]. Propolis was found to inhibit the growth of *C. glabrata, Trichosporon spp, C. albicans, Rhodotorula spp* and *streptococcus* mutants. When the concentration is increased from 20% to 30% in ethanolic removal, action was significant through disc diffusion technique. However, *C. albicans* were not efficient in EEP [106-107].

2.11 Antioxidant Properties

Indian propolis contain antioxidant activity that is due to main chemical substances galangin and pinocembrin. Aqueous extract (AEP) has greater activity contrasted to ethanol extract of propolis (EEP) in antioxidant assays system. It might be because of its greater polyphenols contented. Thus, AEP must be a decent substitute instead of ethanol separate [108-110].

In addition, it very well may be utilized in protection of different free radical related disorders. The Galang showed comparable activity of AEP and EEP and presents higher activity than pinocembrin. This is due to basic structural changes among these two combinations. Additionally, look into is in progress to dissect the constituents of AEP and their antioxidant activity [111].

Its broadened galang in and pinocembrin in the fast making of steady Au and Ag nanoparticles having wide range of exciting types. Beneath the alkaline condition of a given metal particle antecedent, both of the two concentrates were observed in a great degree proficient in combination of Ag and Au nanoparticles [112].

The antioxidant activity of propolis has been observed in topical formulation to stop and treat different diseases like, skin disoders, and aging. The presence of balanced free oxygen radicals, indicate the healthy skinwhile high level of oxygen damges the cell death and also cause aging [113].

Taffine et al. (2017) described by research that propolis found in Algeria region and showed antioxidant activity. The propolis extract presented significantly higher activity than honey. The antioxidant activity of propolis measured by the reduction test which indicated the higher activity in propolis in extracts with methanol, showing the relation of phenolic components and antioxidant activity [114]. In addition, it very well may be utilized in protection of different free radical related disorders. Glang et al. describe by study that activity of EEP shows higher activity than pinocembrin, because of basic structural changes among these combination [115].

2.12 Cytotoxic Activity

The cytotoxic effect of Purtuhuese phenolic extract of different origins was evaluated by using human tumor cells lines(MCF7) breast adenocarcinoma NCIH460 non small cell, lungs carcinoma. The study showed that highly cytotoxic activity for lines of human tumor cells, mostly for HCT15. The study conducted by MTT assay to evaluate the invitro cytotoxicity of Indian propolis againtshuman breast ccancer (CF7), colon cancer, (HCT117)and celllineage of prostate cancer [116]. The propolis extract were incubated with cancer cells linesfor 25 hours, cytotoxicity measured by colometrically and IC_{50} values was calculated. The samples of propolis extract analysis by GC-MS and 44 compounds were identified. The results indicated that inpite of inspite of the differences in the chemical composition of propolis collected from different geographic locations all the samples exhibited significant cytotoxic activity [117].

The Taiwanese propolis consists of two components which is isolated and re structures were clarified primarily by spectral evidence of NMR and found two unreported prenylflavanones given the trivial names propolin A (2) and propolin B. Both propolins inhibit human melanoma, glioma C6 and proliferation of cells HL60 by apoptosis induction. All of the propolis extracts showed concentration dependent DPPH free radical scavenging activity and the ED50 values (mg/ml) for both MeOH and water extracts [118].

The propolis extract of Brazilian and Chinese have strongly antioxident activity free od DPPH as compare the MeoH extract. The IC_{50} value range from 4.8 to 13.8 mg/ml, while extract of MeoH the free radical scavenging activity and IC_{50} value range from 2.9 to 8.36 mg/ml. Yamauchi et al (1994) The effect of propolis from different country like Japan, China, Brazil and USA on methyl linoleate were almost equal. Among the six Brazilian propolis and MeoH extracts had weak scavenging activity than the rest, The difference were small the ED50 value of 4.8mg/ml and B4 (green propolis extract have high quality among all types of propolis [119]. Brazilian propolis showed highly cytotoxicity due to presence of phenolic compound or diterpenoid [119, 120]. Gonzalez et al. (1996) also

studied on Cuban propolis and 85 percent ethanolic extract had showed strongly hepatoprotective activity on injury in rats. Netherlands and Chinese propolis also had potent hepatoprotective activity on DG/TNFinduced cell death. The Dutch and Chinese propolis MeoH extracts possessed good antioxidant activity. The Brazilian propolis like both B3 and B5 showed exhibited DPPH free radical scaven-ging effect and powerful hepatoprotective action [121].

2.13 Use of Propolis in Dermatology

Preparation of propolis has been used in different process like wound healing and regeneration of tissues [122]. It is constant with well-known effects like astringent effect, styptic and propolisattributed effect. In latest studies it has been reported that propolis can be used to treat many dermatological issues (related to skin). In another study it has been reported that the alcoholic solution of propolis, sulphonyl and framykoin has been used in the process of tissue regeneration. Other studies revealed about the efficiency of second degree propolis treatment such as neurodermatitis, microbial eczema and other skin issues [123-125]. For the treatment of skin disorder, the solution of Peruvian balsam, boric acid, arnica extract and acronymic cruris used to cure it. Propolis can be used as alcoholic solution for the treatment of dermatological problems and other pharmacological activities. To check the rabbits hepatic temperature, injection of propolis extract can be used by making propolis 10 to 95 percent alcohol diluted it with water and it increased the temperature of rabbit's hepatic from 0.2 to 0.5 [126].

Atropine and adrenaline correlative studies have been revealed that extract of propolis enhanced the effect of mucous membrane receptors and increased the temperature in the liver and stomach. Extract of propolis as 30% of alcoholic solution used for the treatment of ulcer in comparison of two other drugs. It was reported that some of the efficient effects on the animal model to treat ulcer [27-129]. Rats with ulcer induced with the arsenic pentoxide and caffeine treatment were divided into 4 groups. It has been reported that the propolis aqueous extracts

have a strong effect on the nervous system [130]. The extract of propolis change the normal tension and peristaltic effect of the intestine that is isolated and is result it produce blood vessel vasodilation. Extract of propolis has been reported in the treatment of ear and the infection of respiratory tract. Alcoholic extract with 50% was used for the treatment of chronic suppurative otitis. It was reported that extract of propolis had great effect on the production of tumor cells [131-132].

Extract of propolis act as unspecific agent that enhanced immunogens. Propolis increased different properties of guinea pigs that are immunized like agglutination and precipitating effect [134]. In another study it was revealed propolis extract had great pharma logical effect on the serum agglutinin formation in the immunized calves against the parathyroid [135]. To boost up the synthesis of Nagglutanin and Oagglutinin propolis extract used. To treat the ophthalmic and chronic pharyngitis, alcoholic extract of propolis used in toothpaste and mouthwash to enhance the antiseptic activity by adding 12% of 10% of solution [136-137].

For styptic patent resin's propolis dissolved in the ethanol and solution is cooled at 0'C and then filtered [138]. The waxy filtered with alcoholic has properties like antiseptic, astringent and styptic. Propolis extract tincture with the arnica tincture, chamomile extract and ocresyl salicylate used in the treatment of tooth a gingival disorder. For the treatment of mouth mucosal disease, mixture of glycerol and freons has been added to the propolis [139-140].

By extraction using the organic solvents, propolis preparation with biologically active polyphenol can be obtained. Various pharmaceutical preparations have been described in eastern European. Clinically tested medicinal propolis is Mylit. Propolis extract has been commercially used. Recently, demand for propolis extract commercially has been increased. A colony of bees collects almost 150-200g of propolis per year. There are little amount of propolis available and beekeepers faces many problems. Only a few descriptions available to harvest it from the hives to obtain a product free from the bees wax. A possible use of extract of propolis is suggested by the observation that only 0.05% of aqueous alcoholic propolis

emulsion as supplement to the chicken as basal diet to increase the weight gain by up to 20% [141].

Related to the skin issues as in field of dermatology, Propolis is used for treatment of:

- Wound healing
- Burning
- External ulcer
- Reduction in time of healing
- Enhanced contraction of wound
- Increased process of tissue repair

Perfect synchronized molecular and cellular interactions used to repair the damaged tissues and wound healing [142]. Healing is such a process of biochemical and physiological stages like inflammation, maturation of fibroblasts and tissues. Wound healing process may be described as prototype by using a linear skin wound healing. The starting step at the time of injury is hemorrhage with any wound, formation of fibrin rich clot. Fibrins rich clot stabilize it and produce a scab before dehydration occur. In next step, macrophages soon follow the neutrophils at the injury site and wound debridement by the process of opsonization. The epidermal process responded very quickly then after a day collagen tissue formed between two or three days after the injury, the wound floor is then recovered with the regeneration sheet of epidermal skin [143].

Formation of granulation tissue starts roughly at one with flow and conception of fibroblasts and the starting of capillary production. After 4 to 5 days of wounding, epidermis appeared, fibroblasts actively produced. After this extracellular matrix compound are secreted and the formation of neovascularization start. To initiate this process, proteinases must be secreted by the endothelial cell to lessen the membrane on basement [144].

Without any reproduction of ridges, many components related to the epidermis are rebuilt. After the vascularization process as it matures into the avascular scar tissue. In clinical and other experimental cases, propolis extract used as to treat animal wound. In a study, they observed that by the reduction of inflammatory response, there is betterment in the process of healing of wounds. Propolis extract showed quick healing of the epithelial layer. After the removal of infection, authors explained that propolis proved as useful for the treatment of wound. Process of healing is directly related to the process of inflammation. By increased deposition of collagen fibers it enhanced production of healing molecule [145].

Inflammation that stays for long time can cause necrosis, more damage in tissues and longtime of healing. Properties of regeneration of propolis tissue including healing may be because of the activity that is antioxidant. Free radicals are formed every time; they block the reformation of cell tissues. Normally regeneration of damaged organs can be permitted by the flavonoids by removing the free radicals by propolis extract [146].

2.14 Pharmacological Properties and Toxicity Propolis

Different antimicrobial activity is seen in vitro basically against Gram positive (Staphylococci and Streptococci spp), Gram negative, fungi and viruses. It has been reported in studies that solvent being used for the extraction of propolis is effecting the microbial activity. Antimicrobial activities being seen by oil preparation e.g. Glycerin show inhibition against gram positive bacteria while ethanol and propylene glycol solutions express enhance activity against yeast.

A good synergistic effect seen for Streptomycin and Cloxacilin antibacterial activity while little synergistic effect seen for Chloramphenicol, Polymyxis in media containing standard value of Staphylococcus aureus w4x Strain [147]. A study was conducted for 15 clinical bacterial strains in which the antibacterial activity

was demonstrated along with other factors including inhibition property of water insoluble glucan formation w5x. Arnica extract was compared with propolis resulting in a low activity in 3 conditions [148].

Some infection which are difficult to treat can be treated with propolis ointment e.g. genital HSV infection. Ointment containing propolis not only reduce symptoms of this infection but also being effective in dermatology, w6x dentistry and otorhinolaryngology. Propolis is seen to be effective by its antiflammatory effects on inflammatory models. Propolis also showed inhibitory effect on concentration dependent COX from saline and LPS treated rat lung homogenates [149].

From all the compounds only CAPE and gelangin were on propolis ant inflammation it CAP being more active Propolis also exhibits in vitro immunostimulatory and immunomodulatory effects on w8, 9x macrophages, while in vivo increases the proportion of CD4, CD8 T cells in w10x mice This range of effects could explain why propolis is used in chronic and acute mouth inflammations, periodontist sinusitis, pharingotracheitis or upper and lower airway diseases and skin ulcers w11–13x [150].

Hepatoprotective effects on liver by propolis is seen in rats induced by carbon tetrachloride and w14x paracetamol and allyl alcohol w15x in mice. GSH is antioxidant molecule in liver which is responsible for balancing ormalredox s4 s. it protects against chemical induced injuries.

Proposes inhibits superoxide anion by bmeracaptoethanol autoxidation w17x. CAPE being more active than galangin, CAPE protects against spinal injury. CAPE helps in avoiding the complication during surgical repair of thoracic or thoraco abdominal aortic aneurysms w18x. Anesthetic effect like that of cocaine w19x is seen by propolis along with regenerative effects on cancer cells w10, 2224x on biological tissues w20, 21x and antineoplastic activity [151].

Inhibition of the cell division and the synthesis of protein w25x by propolois reported in recent studies. CAPE was also identified as one of the major active compounds with chemo preventive and antitumoral properties of w24x in propolis.

Although exact mechanism is not clear and need further research and experimentation on it [152, 153]. Propolis is considered safe at low doses: however, adverse effects at doses over 15 gyday are common with the most commonly experienced adverse effects are allergic reactions, and irritations of the skin or mucous membrane. Caution should be used in asthma treatment, and in eczema and nettle-rash patients [154].

2.15 Anti-Protozoal Action

Afterward incubation in the existence of various concentrations of propolis, anti-protozoa action is assessed by an invitro growth inhibitory influence on the culture of parasites [155]. The diseases of humans and animals like Toxoplasmosis, Chagas, Leishmaniasis, giardiasis, malaria and trichomoniasis by the influence of European propolis on protozoal stated by numerous journals. *Trichomonas vaginalis, Toxoplasma gondii, Giardia lamblia, Leishmania donovani* and *Trypanosoma cruzi.* Against the *G. duodenalis* anti-protozoan activity of EEP was stated [156].

2.15.1 Anti-Tumoral Action

The anti-tumoral action for propolis became reviewed. The chemo defensive movement in cell culture and animal models might be going to the result in ability to preclude DNA making in tumor cells, the potential toward provoke apoptosis of tumor cells, and their property to start macrophages to deliver causes in shape for controlling the ability of B, T and NK cells, for my part. Additionally, giving expectation that they will have similar defensive action pastime in human being due to consequences advice that flavonoids from propolis count on a shielding activity against the lethality of the chemotherapeutic specialists or radiation in mice. The mixes with adjuvant most cancers prevention agent remedy may additionally improve the adequacy of chemotherapy with the aid of improving the symptom on leukocytes, liver, and kidneys and consequently empowering dosage acceleration [157], through the caffeic acid. An anti metastatic activity was recognized

phenethyl ester from poplar propolis and also from artepillin C from Baccharis propolis because of this propolis is thought to be effective antitumor agent in various polyphenols [158, 159]. In human lymphocytes, anticarcinogenic capability of propolis in vitro was discovered. Plasma checks had been acquired from 10 sound males, nonsmoking volunteers, which had been incubated and offered to increasing concentrating of propolis (0.01, zero. 05, 0.1, 0.2, 0.5, 0.7, and 1.0 mL) [160]. The suggest micronucleus quotes had been 14.770.38 - 4.0270. 64 Mitotic record costs have been somewhere in the range of 19.4572.22 - 0.2870.33. The contrasts between the manipulate and uncovered cells were statically important (pp; 0: 05). In peripheral human being lymphocytes in vitro are acquaintance to various concentrations of propolis cannot produce a cancer causing influence. Though, it showed that propolis might have a cancer causing influence in high concentrations by increasing micronucleus (MN) rates [161].

2.15.2 Anti-Inflammatory Action

Irritation is the composite biological reaction of vascular tissues to destructive stimuli, such as free radicals, pathogens, damaged cells and irritants. The key influence of the host resistance method is an Anti-inflammatory action [162]. The action of propolis was looked into by Almeida and Menezes, NADPH-oxidase ornithine decarboxylase, Myeloperoxidase movement, tyrosine-protein kinase, and hyaluronidase from guinea pig pole cell shave inhibitory properties of propolis. Through the existence of flavonoids dynamic and cinnamic acid byproducts the anti-inflammatory action can be described [163]. The former comprises of naringenin, quercetin, and acacetin; the later contains caffeic acid (CA) and caffeic acid phenyl ester (CAPE) [164]. Previous incorporates, naringenin, quercetin, and acacetin the last includes caffeic corrosive (CA) and caffeic corrosive phenyl ester (CAPE) [165]. Galangin and CAPE, being average famous propolis components, showed anti-inflammatory action and essentially restrained carrageenan oedema, carrageenan pleurisy, and adjuvant joint pain aggravations in rats [166]. The lipoxygenase pathways of arachidonic corrosive digestion amid aggravation in

vivo are mainly restricted the dietary propolis. The Caffeic corrosive, quercetin, and naringenin were a less intense modulator of arachidonic corrosive digestion than CAPE.

2.15.3 Anti-Diabetic Action

The impact of ethanolic listen of propolis against trial diabetes mellitus-related adjustments becomes inspected. Diabetes was generated in rats by using infusion of streptozotocin (STZ) in measurements of 60 mg/kg in three days [167]. Glucose, lipid profile, blood urea nitrogen (BNU), malondialdehyde (MDA) and urinary egg white was been analyzed. Glutathione, catalase (CAT), malondialdehyde and superoxide dismutase (SOD) were analyzed inside the renal tissue. The wait loss and weight increased in kidney was measured in diabetic rats [168]. Contrasted with the manage everyday rats, diabetic rats had higher blood glucose, BNU, creatinine, add up to cholesterol, triglycerides, low-thickness LDL-C, urinary egg whites and lower high-thickness lipoprotein-ldl cholesterol (HDL-C) tiers. In addition, renal tissue MDA becomes particularly expanded while SOD, GSH, and CAT were essentially diminished. Oral business enterprise of propolis separate in measurements of one hundred, two hundred and three hundred mg/kg between better the frame and weight of kidney, blood glucose levels, lipid profiles, MDA and renal capacity exams. Renal GSH, SOD, and CAT had been altogether increased whilst MDA turned into significantly decreased [169-170]. These observations showed that the propolis can prevent cancer that can enhance oxidative stress and prevent diabetic nephropathy.

2.15.4 Immunomodulatory Action

The immunomodulatory action of water solvent subsidiary (WSD) of propolis was tested. The oral and parenteral business enterprise of the WSD improved the survival price and the suggest survival time in exploratory bacterial and parasitic infections in mice. An elevated competition become watched likewise in Klebsiella

pneumoniae contamination instigated after cyclophosphamide remedy. The WSD empowered peritoneal macrophages to supply in vitro interleukin-1, which related to their lifted aggregate protein emission. What's more, WSD unnoticed to cause lymphocyte multiplication as dictated with the aid of popliteal lymph hub examine. The WSD changed into proposed to increase nonspecific host resistance with the aid of macrophage initiation [171].

2.15.5 Dental Action

The antimicrobial motion of five Propolis test accrued from four locales in Turkey and also from Brazil against the nine bacterial strain that were anaerobic that include Peptostreptococcus anaerobius, Peptostreptococcus micros, Prevotell aoralis, Prevotell amelaninogenica, Porphyro monasgingivalis, Fusobacter iumnucleatum, Veillon ellaparvula, Lactobacillus acidophilus, and Actino mycesnaeslundii became assessed and decided least inhibitory focuses (MIC) and least bactericidal fixations (MBC) of EEP on the development of take a look at microorganisms through making use of agar weak- ening method. All traces were defenseless and MIC esteems ran from four to 512 milligram per milliliter for propolis movement. Propolis of Kazan-Ankara showed pleasant MIC esteems to pondered microbes. MBC estimations of Kazan-Ankara EEP exams ran from 8 to 512mg/mL [172]. Demise become visible inside four hours of brooding for Peptostreptococcus anaerobius, Lactobacillus acidophilus and Actino mycesnaeslundii while being eight hours for Prevotellaoralis, Prevotell amelaninogenica, and Porphyro monasgingivalis twelve hours for Fusobacterium nucleatum and sixteen hours for Veillonell aparvula. It is proved that propolis tests are more compelling against Gram-positive advantageous anerobic microbs than Gram-negative ones. Propolis can be applied in oral cavity due to presence of flavonoids, for instance, pinobanksin, quercetin, naringenin, galangine, chrysin, and fragrant acids, as an instance, caffeic corrosive controlled by using GC-MS exam [173].

Chapter 3

Material and Methods

Material utilized for the research work is given below (Table 3. 1)

Table 3.1: Material Utilized for Research Work

Chemicals	
Methanol	-
Distilled water	-
DPPH reagent(2,2-diphenyl-1-picrylhydrazyl)	-
Ascorbic Acid	-
Terbinafine	-
Streptomycin	-
Nutrient Agar	-
Sabouraud Dextrose Agar(SDA)	-
Brine Shrimps egg,sea salt	-
Eqipments	
Test tubes, Falcon tubes 15ml, 50ml	Eppendorf tubes
Vials	Beaker 100ml, 500ml, 1000ml
Micropipette	Test tubes racks
Cotton plugs	Discs
Cotton swabs	Para film or masking tape
Aluminum Foil	Forceps

3.1 Microorganisms Used

TABLE 3.2: List of Microorganisms Used

Microorganisms Used	
Bacillus subtilis	
AT-10	*Aspergillus fumigatus*
Staphylococcus aureus	*Aspergillus niger*
Enterobacter aerogenes	*Mucor Species*
Micrococcus luteus	*Fusarium solani*

3.2 Research Methodology Outlines

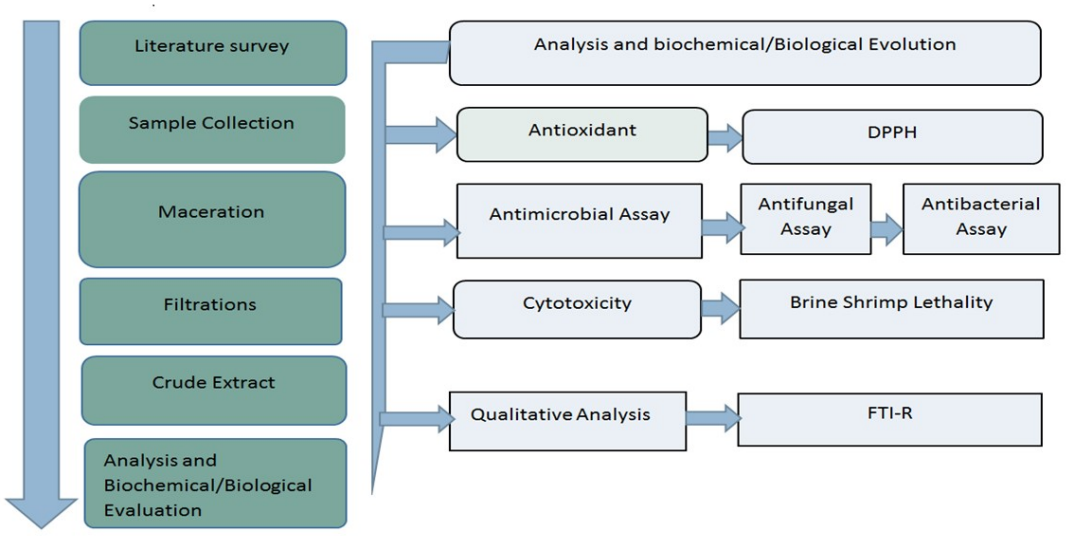

FIGURE 3.1: Shows the detail outlines of our research methodology

3.3 Samples Collection

In the recent study, four different types of Propolis samples were collected from different areas of Pakistan. One of the Propolis sample was collected Islamabad (Kallar Syedan) and tagged as Propolis 1, 2nd Propolis sample was collected from

the hives of Honey Research Institute of NARC (National Agriculture Research Centre) and was tagged as 2. And third Propolis was collected from Nowshera and tagged as Propolis 3. And fourth Propolis was collected from Lahore which was commercialy processed form and tagged as Propolis 4. All the Propolis samples were in dried form, properly kept at refrigerator at 4℃.

3.4 Extraction

The technique employed for extraction was manual maceration. Precise weighed (10gm) of Propolis samples were crushed into small pieces and extraction was performed in 70 percent methanol in 100ml. And it was left at room temperature overnight. The suspnsion was filtered on the second day and the extract of Propolis was kept in refrigerated at 4℃ [174].

3.5 Biological Evaluation of Propolis Extract

3.5.1 Antioxidant Assays

Antioxidant activity of Propolis samples was determined by using DPPH method (2, 2-diphenyl-picryl-hydraxyl-hydrate) that was described by Khan et al. (2015) [171, 175].

3.5.1.1 Sample Preparation

Different dilutions were used for antioxidant assays (10, 20, 30μM) with the adding of distilled water in the Propolis extract. Preparation of DPPH Solution (Free Radical Scavenging Assay; FRSA). To prepare DPPH solution freshly, 0.12 mg of DPPH was added in 100 ml of methanol. Ascorbic acid solution was prepared with 1 ml of DMSO added to 1 mg of ascorbic acid. Stock solution of 4mg/ml was prepared in methanol for each test extract.

3.5.1.2 Procedure

Free radical extinguishing capability of extracts or samples is assessed by DPPH reagent based assay. A change in absorbance values is detected because antioxidant in test samples cause production of hydrazine which reduces the discoloration of purple color of DPPH reagent.

Spectrophotometer was used for this assay and whole procedure was run in triplicate. From each stock solution, tested Propolis sample (200μl) was taken and transferred to respective vial in the microtiter plate followed by the addition of DPPH reagent (3ml). For 60 minutes, then incubated the resultant mixture at 37℃in a pitch dark surrounding and measured absorbance at 518 nm with the help of spectrophotometer and % scavenging activity of each Propolis sample was find Out by given formula:

$$\%age\ scavenging = \left[1 - \frac{Abs}{Abc}\right] \times 100 \tag{3.1}$$

Where, Abs is Absorbance of sample containing DPPH reagent, Abc is Absorbance of negative control containing Distilled water and DPPH reagent. Standard ascorbic acid was employed as positive and distilled water as a negative control.

3.5.2 Antimicrobial Assays

There are two kinds of antimicrobial assays were executed to evaluate the biological activity of Propolis extract. Antibacterial assays Antifungal assays

3.5.2.1 Antibacterial Assays

For antibacterial assessment five strains of bacteria were used. Antibacterial properties of Propolis extract were examined as described by Khan et al [176] using the method of disc diffusion.

3.5.2.1.1 Bacterial Strains Used

3.5.2.1.1.1 Gram Positive Strains

Staphylococcus aureus

Micrococcus luteus

Bacillus subtilis

3.5.2.1.1.2 Gram Negitive strains

Salmonella arunes

Enterobacter aerogenes

E. coli

3.5.2.2 Preparation of Sample

The 10 mg/ml stock solutions of all Propolis extracts were prepared in 100ml of Methanol. And in this assay different dilution of this stock were used (10ppm, 20ppm, 30ppm). Streptomycin (positive standards) stock solutions (100ppm) were prepared.

3.5.2.3 Inoculum Preparation

The culture was refreshed by taking 10 ml aliquot of sterile nutrient broth inoculated with sterile loopful of bacterial colonies maintained at 37℃ for 24 hrs.

3.5.2.4 Media for Bacterial Growth

Nutrient Agar was used in petriplates for bacterial production. In 1 liter of distilled water, add 28g nutriant agar. Nutrient Agar is composed as below

Peptone	6g/500ml
Yeast Extract	4g/500ml
Agar	15g/500ml

Sodium Chloride	4g/500ml
Distilled water	3 liter

3.5.2.5 Procedure

Refreshed bacterial cultures was used to prepare lawn on Nutrient Agar petri plates. By taking 50 μl aliquot from 24 hrs. Four of each Propolis extract was infused on discs of filter paper (sterilized) of 10, 20 and 30ppm concentration and then placed on properly labeled seeded agar plates.

Streptomycin were also infused on discs and placed on plates as One of positive control. Distilted water was use as a negative control. Incubation was done at At 37℃for 24 hrs. Around each disc (Propolis samples + control) zone of inhibition was examined, measured in milli meters (mm) with vernier caliperand then recorded. The assay was run as triplicate analysis.

3.5.3 Antifungal Assay

For determining the antifungal activity of Propolis extract, Tube dilution method was used [177].

3.5.3.1 Preparation of Sample

To make 20 mg/ml solutions, Accurately weighed 10 mg test extracts were dissolved in 100 ml of Methanol. Stock solution of standard drug Terbinfine was prepared.

3.5.3.2 Inoculum Preparation

Spores of fungal strains was collected from stock cultures on sterile SDA plates. At 28℃, incubation of plates was done for 7 days.

3.5.3.3 Preparation of Media for Fungal Growth

For the fungal growth Sabouraud Dextrose Agar was prepared. Its composition is given below:

Sabouraud Dextrose Agar	26g/400mL of distilled water.

3.5.3.4 Use of Fungal Strains

Four strains of fungus were used for the antifungal assays.
Aspergillus flavus
Aspergillus fumigatus
Aspergillus niger
Mucor Species

3.5.3.5 Procedure

Antifungal assay was carried out as shown previously by [176]. 10 cm Mark test tubes. Add (5ml) have been swabbed with sterile sabouraud dextrose agar. Refreshed with 100μl inoculum and make slants. Cover test tubes with plugs made from cotton. Place the Terbinfine (Positive Standard) and Negative Standard on test tubes. Incubation was done 37℃ for 4 days. Vernier Caliper has measured fungal growth on tubes. The assay was run as triplicate analysis. Following formula was used to calculate the percentage

$$\%age\ Viability = \left[\frac{(-ive\ control) - (Test)}{-ive\ control}\right] \times 100 \tag{3.2}$$

3.5.4 Cytotoxicity Assays

Brine shrimps cytotoxic assay was completed to determine the level of toxicity of Propolis extract as reported earlier [177-178].

3.5.4.1 Preparation of Samples

All Propolis extracts were prepared with 10 mg/ml stock solutions in100ml methanol. Standard stock solution for the drug doxorubicin was prepared as 4mg/ml

3.5.4.2 Sea Salt Preparation

Simulated sea water was prepared by dissolving sea salt (34g) in 1 liter of distilled water.

3.5.4.3 Hatching of Eggs

Brine shrimps eggs were hatched in sea salt water ($34gL^{-1}$).

3.5.4.4 Procedure

The preliminary cytotoxicity of crude extracts to larvae of brine shrimp (Artenia salina) was determined by a 24-hour lethality test as previously described by [179]. The Artenia salina eggs were hatched in a specially designed perforated biocompartment tank filled with simulated sea water.

The compartment containing eggs was completely covered with aluminium foil while other was lightened with a light source. The eggs were hatched and nauplii started moving towards the lightened compartment of the tank through small perforations, After specified incubation period. With Pasteur pipette, the hatched nauplii were then collected and placed in a beaker containing sea water.

Two fold serial dilution of test extracts was made up to the final concen trations (1000, 500, 250 μM). 15 mature nauplii were transferred and 150 μl of sea water was added to each vial. After incubating at 25℃ for 24 hrs, dead nauplii were counted using pasture pipette (3X magnifying glass). The whole experiment was performed thrice. The percent lethality of each extract was determined using

formula:

$$\%age\ Mortality = \left[\frac{(No.\ of\ AS\ in\ -ive\ control) - (No.\ of\ AS\ in\ test)}{No.\ of\ AS\ in\ the\ -ive\ control}\right] \times 100 \tag{3.3}$$

3.5.5 Qualitative Analysis

Mainly, two tools/techniques were used to determine the and structures of organic molecules and functional groups present in our test extracts. These two tools are GCMS and FT-IR.

3.5.6 Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

FT-IR technique indicates the bonds existed in the compound and consequently be used to determine functional groups of the molecule.

Chapter 4

Result and Analysis

4.1 Biological Evaluation

4.1.1 Antioxidant Activity (DPPH Assays)

Antioxidant potential of Propolis was assessed by DPPH assay. Free radical scavenging activity was showed by different Propolis samples. In present study Propolis showed grater antioxidant activity. Stability and accessibility within the cells make a properly managed DPPH free radical. To check the potential for scavenging in test extracts and, consequently, antioxidant capability. The DPPH reagent has a dark purple color and is capable of obtaining an electron from donor antioxidants, resulting in a color change from dark purple to light purple to light yellow. This decoloration is due to the presence of antioxidants in Propolis extracts which can be quantified by a spectrophotometer calculating changes in the absorbance values at 517 nm. The potential free radical scavenging activity of all the Propolis extracts was de termined by DPPH test (Figure 4.1). The results of DPPH assay open that significant percentage of free radical Scavenging was higher observed in Propolis 2 than Propolis 1, 3, 4 with the value of 84 $\pm$ 0.1 and IC50 value is 45.0 and Propolis 1, value of 45 $\pm$ 0.1 and IC50 value is 19.0 and the value of Propolis 3, 61 $\pm$0.1 and IC50 value is 33.And Propolis 4, value of 55 $\pm$ 0.1 and IC50 value is

28 at 30 concentrations concentrations respectively and % scavenging of Propolis 2 in term of IC50 and P-value is < 0.001 was higher significance than Propolis 1, 3, 4. The % scavenging of all the Propolis samples were as follows [Table 4.1]. The free radical scavenging activity ofall the active samples in terms of % scavenging and IC50 followed in the order:

Propolis 2 (NARC) > Propolis 3 (Nosheraw) > Propolis 4 (commercially processed form Lahore) > Propolis 1 (Islamabad)

The characterisation of Propolis extracts in vitro was found using DPPH assay based on the scavenging of stable free radicals.In the DPPH assays, % scavenging of Propolis 2 in term of IC50 and P-value is < 0.001 was higher and significance than Propolis 1, 3, 4 which might be described to the different functional groups present in Propolis 2 extract as confirmed by FTIR analysis.

Also confirmed from previous findings and the reason could be some factor like geographical regions, flora natural climate conditions, time periods for cultivation and processing, moisture and storage.Our results are in close agreement with the prereported work where Propolis from different geographical orogin showed [180]. In general, phenolic compounds belonging to substances expressing the ability to scavenge free radicals are primarily responsible for the antioxidant capacity of bee products. Bee products prevent agricultural induced oxidative damage in fish.

They consist of two main groups of compounds flavonoids and phenolic acids Bee pollen polyphenols: structure, absorption, metabolism. Argentina, Australia, Brazil, Bulgaria, Chile, China (Hebei, Hubei, and Zhejiang), Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, the United States, and Uzbekistan were compared with Propolis antioxidants of various geographical backgrounds [181].

The Bcarotene bleaching and 1, 1diphenyl, 2 picrylhydrazyl (DPPH) free radical scavenging assay systems have prepared and evaluated ethanol extracts of Propolis (EEP) for the antioxidant activities of EEP samples. Proplis had relatively strong antioxidant activities in Argentina, Australia, China, Hungary and New

Zealand, and was also associated with the total polyphenol and flavonoid content. Propolis with resilient antioxidant activity contained antioxidant compound like phenethyl caffeate, kaempferol. The antioxidant activity of Propolis varying geographical origin. Different products from a unique Propolis extract obtained using various solvents such as hydroalcoholic, glycolic (98% propylene glycol) and glyceric solutions and oil have been evaluated for chemical composition in powder form called ESIT12. The antioxidant properties of the four preparations were determined, the activity was similar among them, thus revealing that it is strictly related to the content of polyphenols for Propolis products whose composition is quite comparable.

TABLE 4.1: Values of Absorption and % Scavenging of selected Propolis extracts.

Antioxidant Assays			
Samples Names	**Concentration(μgml)**	**%Scavenging**	**IC50(μg/ml)**
Propolis 1	10	10 ± 0.1	19
	20	44 ± 0.5	
	30	45± 0.1	
Propolis 2	10	26 ± 0.3	45
	20	52 ± 0.54	
	30	84 ± 0.1	
Propolis 3	10	11 ± 0.1	33
	20	46 ± 0.5	
	30	61± 0.1	
Propolis 4	10	13 ± 0.1	28
	20	42 ± 0.5	
	30	55± 0.1	
Positive control	10	22 ± 0.33	16
	20	42 ± 0.55	
	30	68 ± 0.1	
Negative control	0	0	0

TABLE 4.2: Analysis of Variance for Factors Affecting the Free Radical Scavenging Activity of Crude Propolis Extract.

Source of Variation	**Sum of Squares**	**Df**	**Mean Square**	**F-Value**	**P-Value**	**Sign**
Interaction	189.6	4	94.7	6.316	<0.0001	Yes
Types of Propolis	580.1	4	597.1	26.38	<0.0001	Yes
Conc.	143.30	4	662	297.27	<0.0001	Yes
Residual	235.6	12	22.78			

4.2 Antimicrobial Potential

4.2.1 Antibacterial Activity

Antibacterial potential tested by disc-diffusion method showed significant activity against the bacterial strains employed in terms of zone of inhibition (mm ± SD) as shown in table 4.3. By comparing results with control treatments, the antibacterial potential of Propolis samples was determined. Minimum inhibitory concentration was determined against the di erent concentrations of the Propolis sample, including 10ppm, 20ppm, 30pp, and Minimum Inhibitory Concentration (MIC), which is the lowest concentration of any chemical or drug leading in a delay in bacterial growth. After an incubation period of 24 hours clear zone of inhibition was observed. Propolis 1 showed maximum activity against Staphylococcus aureus (0.4 ± 0.1mm) and *salmonella arunes* (0.3 ± 0.1mm). The weakest activity of Propolis 1 was observed against *E.coli* 0.019 ± 0.01mm respectively. In table 4.3, 0,-, = shows No activity, Propolis 2 showed maximum activity against *E.coli* (0.1 ± 0.4 mm) and Staphylococcus aureus (0.2 ± 0.8mm). The weakest activity of Propolis 2 was observed against B.subtilis(0.21 ± 0.1) table 4.3. Propolis 3 showed maximum activity against. Staphylococcus aureus (0.5 ± 0.1mm) and E.aerogenes (0.3 ± 0.1mm). The weakest activity of Propolis 3 was observed against salmonella arunes i.e. 0.017 ± 0.01mm respectively table 4.3.

Table 4.3: % Inhibition against bacterial strains of selected Propolis extracts

Samples at different Concentration (ppm)	**Antibacterial Assay Diameter of zone of inhibition in mm (Mean ± SD) * (MIC: µg = ml)**								
	Staphylococcus aureus	MIC	*E.aerogenes*	MIC	*s.arunes*	MIC	*B.subtilis*	MIC	*E.coli*
				Propolis1 Islmbd					
10 %	0.06 ± 0.1	100	0.06 ± 0.01	100	0.1 ± 0.01	100	0	100	0.01 ± 0.01
20%	0. 1± 0.1	100	0.04 ± 0.01	100	0.12 ± 0. 5	100	0.133 ± 0.01	100	0.01 ± 0.01
30%	0. 4± 01	100	0.2 ± 0.1	100	0.3 ± 0.5	100	0.14 ± 0.01	100	0.019 ± 0.01
Positive Control (Streptomycins)	2± 0.01	100	2.3 ± 0.07	100	3.0 ±0.01	100	3.5 ± 0.01	100	4.0± 0.05
Negative Control	-		-		-		-		-
				Propolis2 NARC					
10%	0.04 ± 0.01	100	0	100	0.1 ± 0.1	100	0.06 ± 0.01	100	0.012 ± 0.01
20%	0.04 ± 0.01	100	0.03 ± 0.01	100	0.2 ± 0.1	100	0.33 ± 0.1	100	0.166 ± 0.01
30%	0.2 ± 0.8	100	0.12 ± 0.1	100	0.13 ± 0.5	100	0.21 ± 0.03	100	0.1 ± 0.4
Positive Control	2 ± 0.01	100	2.3 ± 0.05	100	3.5 ± 0.01	100	3.7 ± 0.01	100	4.0± 0.05
Negative Control	-		-		-		-		-

TABLE 4.4: % Inhibition against bacterial strains of selected Propolis extracts.

Samples at different Concentration(ppm)	**Antibacterial Assay** **Diameter of zone of inhibition in mm (Mean ± SD) * (MIC: µg = ml)**								
	Staphylooccus aureus	MIC	*E.aerogenes*	MIC	*s. arunes*	MIC	*B.subtilis*	MIC	*E.coli*
				Propolis3 Nowsheraw					
10%	0.1 ± 0.1	100	0	100	0.1 ± 0.01	100	0.06 ± 0.01	100	0.01 ± 0.01
2o%	0.1 ± 0.1	100	0.06 ± 0.01	100	0.12 ± 0.01	100	0.133 ± 0.01	100	0.01 ± 0.01
30%	0.5 ± 0.5	100	0.3 ± 0.01	100	0.017 ± 0.4	100	0.136 ± 0.01	100	0.01 ± 0.01
Positive Control (Streptomycins)	2± 0.01	100	2.5 ± 0.05	100	3.0 ±0.01	100	4.0 ± 0.01	100	3.5 ± 0.05
Negative Control	-		-		-		-		-
				Propolis4 Lahore					
10%	0.06 ± 0.01	100	0.01 ± 0.01	100	0.1 ± 0.1	100	0	100	0.012 ± 0.01
20%	0.06 ± 0.01	100	0.03 ± 0.01	100	0.2 ± 0.1	100	0.33 ± 0.1	100	0.166 ± 0.01
30%	0.22 ± 0.6	100	0.1 ± 0.1	100	0.6 ± 0.1	100	0.4 ± 0.01	100	0.56 ± 0.1
Positive Control	2 ± 0.01	100	2.5 ± 0.05	100	3.0 ± 0.01	100	4.0 ± 0.01	100	3.5 ± 0.05
Negative Control	-		-		-		-		-

Table 4.3, shows No activity, Propolis 4 showed maximum activity against salmonella arunes (0.6 ± 0.1 mm) and B.subtilis (0.4 ± 0.1mm). The weakest activity of Propolis 4 was observed against Staphylococcus aureus (0.22 ± 0.6) table 4.4.

The results of our study are the inhibitory effect of Propolis against K.pneumonia manifested in harmony. *E.coli* and pneumonia [182, 183]. Propolis phenolic compounds can contribute to an antimicrobial activity, resulting in the release of intracellular membrane components such as amino acids, proteins, pentose and phosphates leading to membrane disruptions and permeabilityand also inhibited lipid peroxidation [184]. These findings confirmed the results of our study that Propolis has antibacterial potential against vast domains of gram positive and gram negative bacteria.

The standard Streptomycin antibiotics were used as positive control and showed minimal antibacterial potential against bacterial strains, as shown in table 4.3. The use of distilled water as negate tested. The results of our research work confirmed the presence of different bioactive compounds in our tested extracts that might contributed to antibacterial activity as FTIR analysis.

4.2.2 Antifungal Activity

Antifungal species are profitable agents for a variety of diseases and resist the pathogenic fungal species. There is age requirement to assemble such alternatives that may be useful against humanity's problems. For this reason, medicinal products are synthesized from natural plants with many antibiotic properties, such as antioxidant, antibacterial and antifungal activities, which are of great use [185]. All of the extract of strains was found to have significant antifungal activity, the standard antifungal drug (Terbinafine) and its final concentration used was 10 µg/disc. The maxi mum percentage of zone of inhibition of fungal strains of Propolis 2 is higher than Propolis 1,3,4 i.e Fusarium solani was 79 ± 0.1mm and and Propolis 1, was 43.3 ± 0.1mm and Propolis 3, was 62.2 ± 0.1mm Propolis 4, was 51 ± 0.1mm respectively. The Minimum percentage of zone of inhibition

of Propolis 1 and Propolis 2 i.e. As pergilus niger was 29± 0.01mm and 16 ± 0.01mm Propolis 3 and Propolis 4, 21 ± 0.01mm, 19± 0.01mm respectively,the assay was run as triplicate analysis [186]. The percentage of inhibition against Fungal strains of selected Propolis extracts:

Propolis 2 (NARC) > Propolis 3 (Nosheraw) > Propolis 4 (commercially processed from Lahore) > Propolis 1 (Islamabad)

TABLE 4.5: % Inhibition against Fungal strains of selected Propolis extracts.

S.No.	***Fusarium solani***	***Aspergilus mucor***	***Aspergilus niger***	***Aspergilus fumigants***
Propolis 1	43.3±0.1	44±0.1	16±0.01	31±0.01
Propolis2	79.3±0.1	48±0.4	29±0.01	38±0.01
Propolis3	62.2±0.1	41±0.4	21±0.01	31±0.01
Propolis4	51±0.1	47±0.4	19±0.01	33±0.01
Positive Control	100	100	100	100
Negative control	0	0	0	0

4.2.3 Cytotoxicity Assay

4.2.3.1 Brine Shrimp Lethality Assays

To observe the cytotoxic effect of the extract of Propolis samples a lethality assay was performed on brine shrimps. Various concentrations were used for plant extracts i.e. 300ppm, 200ppm and 100ppm and showed significant toxic effects. Toxicity has been observed to decrease with decrease in concentrations.The earliest cytotoxicity of the Propolis against Arternia salina nauplii (brine shrimp larvae) was assessed and the results obtained were analyzed for the determination of lethality profile of the selected Propolis by employing the brine shrimp Lethality [186]. The results are shown that Propolis 2, has maximum cytotoxicity and

significant with percentage mortality of 98.66 ± 0.01 IC50 value of 230 μg/ml and p-value is < 0.001, followed by Propolis 1 with percentage mortality of 53.66 ± 0.01, IC50 value of 128 μ g/ml and p-value is < 0.001, followed by Propolis 3 with percentage mortality of 77.66 ± 0.01, IC50 value of 180 μ g/ml and p-value is < 0.001, followed by Propolis 4 with percentage mortality of 61.66 ± 0.01, IC50 value of 145 μ g/ml and p-value is < 0.001, at 300 μg/ml concentration. The results are shown that Propolis 2, respectively table 4.6.

The cytotoxic potential of the Propolis extracts arranged in the following manner:

Propolis 2 (NARC) > Propolis 3 (Nosheraw) > Propolis 4 (commercially processed form Lahore) > Propolis 1 (Islamabad)

The viability of shrimps was observed to have decreased considerably as the higher concentration and had a higher mortality rate than lower concentrations of Table 4.5 of the Propolis extract. Brine shrimps or *Arternia salina* larvae and carcinoma cells of mammals are commonly deduced to behave same in many way, which is why the cytotoxic effects of the test extracts undertaken might be become potential candidates for antitumor and anticancer activities; possible biological activities can be tested against malarial parasites, pests, tumors and harmful microbes. Sample activity was based on dependent concentration and as concentration of samples decreased, the percentage (percent) mortality rate also decreased confirmed the previous studies by using the larvae of brine shrimps as a test model [187].

Bangladesh's Propolis antioxidant, cytotoxic, and antinociceptive activities were conducted. Bangladash Propolis contained higher levels of polyphenols, flavonoids, tannins, ascorbic acid vitamin E and sugar reduction compared to previous reports of multicountry Propolis.

BDP also exhibited higher free radical scavenging activities and a dosedependent power reduction activity indicating its superior potential for antioxidants. In addition, BDP extract was most toxic to brine shrimp nauplii with a lethal concentration of 57.99 μg/mL (LC50) of 50 percent study was conducted to assess the cytotoxicity of extracts from Propolis.

TABLE 4.6: Brine shrimps lethality potential of selected Propolis extracts.

	Cytotoxicity Potential		
Samples Names	**Conc(μgml)**	**%Mortality**	**IC50(μg/ml)**
Propolis 1	1000	53.66±0.01	128
	500	62±0.01	
	250	50±0.01	
Propolis 2	1000	98±0.01	230
	500	90±0.01	
	250	81±0.01	
Propolis 3	1000	77.66±0.01	180
	500	64±0.01	
	250	55±0.01	
Propolis 4	1000	61.66±0.01	145
	500	67±0.01	
	250	52±0.01	

Sirindhornae squamous cell carcinoma (HNSCC) cell lines against two heads and necks. T. Propolis produced by sirindhornae exhibits cytotoxic effects on HNSCC cells. In addition, DMEPB and DMEPC differently inhibited the proliferation of a metastatic HNSCC cell line.

TABLE 4.7: Analysis of Variance for Factors Effecting the Viability of Brine Shrimps

Source of Variation	**Sum of Squares**	**Df**	**Mean Square**	**F-Value**	**P-Value**	**Sign**
Interaction	1239	4	233.2	21.02	<0.001	Yes
Types of Propolis	37620	4	8207	966.8	<0.001	Yes
Concentration	2198	1	1399	150.5	<0.001	Yes
Residual	354.9	13	13.62			

4.2.4 Qualitative Analysis

4.2.4.1 Determination of Functional Groups using FT-IR Spectroscopy

The most commonly used technique for identifying functional groupings is FTIR spectroscopy. FTIR spectroscopy is a quick, useful and responsive technique used to illustrate the Structre and physiochemical properties of the material under investigation [188].

Table 4.8: FT-IR Analysis of Propolis 2 Extract (NARC)

Sr.#	**Freq of band (cm-1)**	**Experimental Freq of Propolis (cm-1)**	**Bond**	**Functional groups**
1	3600-3300	3311.12P2, 3311.12 P2	O-H Stretch, H-bonded	Alcohols, Phenols
2	3000-2850	2943.80P2	C-H Stretch	Alkanes
3	3300-2500	2831.63P2 2849.63P2	O-H Stretch	Carboxylic acid
4	1740-1720 1245-1025	1639.10P2 1639.10P2 115.12P2 116.13P2	C=Stretch C=N Stretch	Aldehydes, Saturated aliphatic
5	1750-1680	P2	C=O Stretch	Carboxylic acid
6	1760-1665	P2	C=OStretch	Carbonyls (general)
7	1720-1666	P2	C=O Stretch	Unsaturated aldehydes, Ketones
8	1670-1640	P2	-C=C-Stretch	Alkenes
9	1640-1570	P2	N-H Bend	1 amines

10	1540-1465	P2	N-O Asymmetric stretch	Nitro compounds
11	1700-1100	1449.62 P2 1o30.51 P2	C – C Stretch	Aromatics
12	1480-1260	1269.84P2	C –H Rock	Alkanes
14	1300-1150	1115.11P2 1115.1P2	C-H Wag	Alkyl halides
15	1250-1020	1115.11P2 115.11P2	C-N stretch	Aliphatic amines
16	1320-1000	1021.61P2 1021.62P2	C-O Stretch	Alcohols, acids, Esters, Ethers
17	1000-650	615.06P2 600.05P2	C-H Bend	Alkenes
18	860-650	578.67, 531.13P2	C-Br Stretch	Alkyl halides
19	580-525	569.16, 543.47, 522.10P2	C-Cl Stretch	Alkyl halides

Functional groups can be detected in this technique depending on the composition of the extract and also the solvent polarity. The characterization of crude extracts of Propolis.The present study confirmed the presence of functional groups identified by analysis of the FTIR spectroscopy. Figures and table showed the infrared spectrum of each Propolis and characteristic bands ranging from 4000 cm1 to 515 cm^1 in all sample spectrum of Propolis were observed.

The results summarized in table 4.7 illustrate the presence of the highest absorption band in the Propolis region of 3500-3200 cm1. This band is caused by the presence in hydration water of alcohol and phenolic groups, andor the H bonded O-H stretch. It means Propolis that has hygroscopic characteristics and shows hydrophilic character. Saturated CH hydrocarbons stretch under 3000 cm^{-1}. The

strong bands appear in the Propolis 2 indicated the stretching of C-Cl and C-Br in alkyl halides at 860 cm^{-1} to 650cm^{-1} and 580cm^{-1} to 525-cm^{1}.

Another strong absorption band was also observed at 2849 cm1 due to OH stretching, indicating the presence of carboxylic acid in all the proporlis. The strong bands appear in the proporlis 2 indicated the stretching of C-Cl and C-Br in alkyl halides at 860 cm^{-1} to 650cm^{-1} and 580cm1 to 525cm^{1} [189]. Another strong absorption band was also observed at 2849 cm-1 due to OH stretching, indicating the presence of carboxylic acid in all the- Propolis Conjugation plays a important role in the observation of carbonyl frequency for the functionality of double bond. The band in Propolis between 1700 cm^{-1} and 1100 cm^{-1} indicated the presence of aromatic compounds that contributed to the antioxidant and other Propolis biological activities [189-186] supports the confirmation of our table of results 4.5.

The other strongest band was also observed at 1030 cm1 confirmed the presence in our all test extracts of esters, carboxylic acid, ether and alcoholic compounds, which also proved their strong aroma, taste and these compounds play significant roles in Propolis bioactivity. Many small peaks between 1470 cm^{-1} 1260 cm1 and 970 cm^{1}, 522 cm^{1}, were observed, confirming the presence of many functions. Similar results were obtained in previous research work that also showed O-H (alcohols, phenols), C-H (aliphatic), C=O (carbonyl), C-O-C (esters), C-N (aliphatic amines). These previous findings precisely coordinate with the present results justifying our perspective.

Present research work regarding FT-IR evaluation of Propolis is in favor of all elements as the particular bands demonstrate the presence of aromatic and organic compounds, reconfirmed the antioxidant and other biological activities of selected Propolis extracts. So it was clear from table and spectra of these Propolis samples that there were many similarities related to functional groups of these Propolis. support the result of our study for different biological activities.

These results of Propolis have shown that the extracts of these Propolis could be safely used in pharmacy and other industries as well. Different products were

evaluated for chemical composition from a unique Propolis extract obtained using various solvents such as hydroalcoholic, glycolic (98 percent propylene glycol), and glyceric solutions, and oil, as well as in powder form, called ESIT1. Overall, flavones and flavonols in the glyceric extract ranged from 20% to 36%, while flavanones and diidroflavonols ranged from 28% to 41%. Glycolic and hydroalcoholic extracts were found to be richer in the total content of polyphenols, in addition to their quite similar composition (Chemical Composition and Antioxidant Activity of Propolis Prepared in Different Forms and in Different Solvents Useful for Finished Products).

4.2.4.2 Biochemical Analysis of Samples via FT-IR

The significant spectral range present between 3500 to 515 cm-1 gives the way to distinguish different Propolis and the all the organic compounds found in these Propolis extract that contribute to significant biological roles with different com positions [189]. In the present study, a novel effort has been made to correlate the functional groups present in Propolis extracts and phytochemical and different biological activities manifested by these extracts.

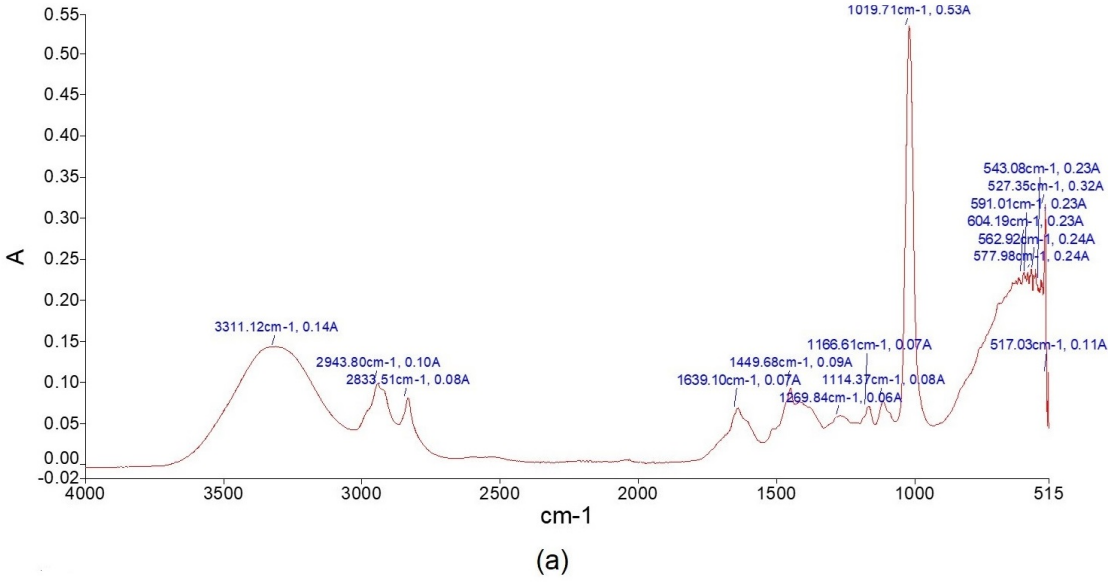

FIGURE 4.1: Absorption spectra of Propolis 2. FT-IR spectrum of Propolis 2 showing significant functions groups for phytochemical, antioxidant, antimicrobial, cytotoxicity activities

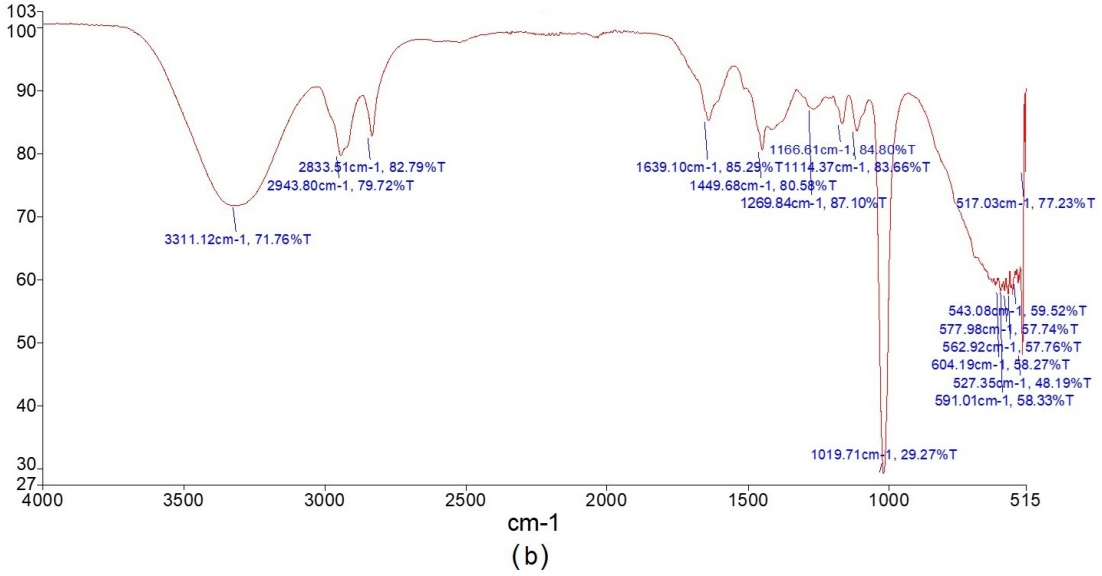

FIGURE 4.2: Transmission spectra of Propolis 2. FT-IR spectrum of Propolis 2 showing significant functions groups for phytochemical, antioxidant, antimicrobial, cytotoxicity activities

Chapter 5

Conclusions and Recommendations

Propolis is a natural product being investigated against pathogens and also against organisms that cause acquired infections in the community. Resistance to antimicrobials leads to increased costs of disease, death and health care, highlighting the need for new antimicrobials. Current research has focused on the use of old medicine/natural products to manage and control diseases. Resistance has appeared in opportunistic microorganisms, alongside the well-known pathogens. Propolis is non-toxic and shows a wide range of antimicrobial activity against different microorganisms.

Propolis is widely used in folk medicine, and various tests have shown that Propolis is antibacterial, antiviral and antifungal. In conclusion, four different propolis samples were collected from different areas of Pakistan. One of the Propolis sample was collected Islamabad (Kallar Syedan) and tagged as propolis 1, 2nd Propolis sample was collected from the hives of Honey Research Institute of NARC (National Agriculture Research Centre) and was tagged as 2. And third propolis was collected from Nowshera and tagged as Propolis 3. And fourth propolis was collected from Lahore which was commercialy processed form and tagged as propolis 4. The maximum antioxidant aptitude reported as the equivalent of ascorbic acid

was also calculated to be the highest in propolis 2 extract whereas propolis 1, 3, 4 extract showed less antioxidant Potential. In antibacterial testing, all propolis extracts were active against five bacterial strains tested which confirmed their use and effectiveness against various infections. Among them, remarkable activity was shown against *Salmonella arunes, Staphylococcus aureus*, by Propolis 1 and Propolis 2 extracts however; modest activity was observed against *Staphylococcus aureus* and *E. coli.* followed by propolis 3,activity was *Staphylococcus aureus*, *E. aerogene* and propolis 4 activity was *Salmonella arunes, B. subtilis* by all tested samples. Least antibacterial activity was observed by Propolis 1. Subjected Propolis samples showed maximum antifungal activity was observed by Propolis 2 followed by Propolis 1, 3, 4 against the fungal strains tested in our study.

The cytotoxicity profile established using the lethality assay of brine shrimps confirmed the highest efficacy of Propolis 2 extracts which may be proposed for use as anticancer and anti-mutagenic agents while minimal activity was observed in Propolis 1, 3, 4. Our results provide clear evidence that despite the large differences in the chemical composition of propolis from different geographic locations, all samples show significant antibacterial and antiviral (and most of them antiviral) activity. This is an expected result, as propolis is believed to be bees defense against infections. Our results, as well as the literature data on the chemical composition and biological action of propolis, can not indicate a particular substance or class of substances that could be responsible for this action. Obviously different combinations of substances are essential for the biological activity of bee glue in different samples. It is important to note that all investigations on the antibacterial action of individual substances, isolated from propolis, have shown that not one single component of propolis has an activity greater than that of the total extract (Kujumgiev et al., 1993; Serra Bonvehi et al., 1994). It seems that propolis' chemical properties are not only beneficial to bees but also have general pharmacological value as a natural mixture and not as a source of new potent individual antimicrobial, antifungal and antiviral compounds. All the extracts tested for propolis confirmed the presence of significant functional groups identified by the analysis of FT-IR spectroscopy. The results of our detailed screening led us to

the conclusion that the Propolis investigation revealed the additional benefits of these Propolis and showed promising prospects for the discovery of new bioactive molecules. Results have shown that this Propolis extracts could also be used safely in pharmacy and other industries.

5.1 Future Prospects

- Bioactivity guided isolation should be the most logical extension of our study in order to isolate,identify and characterize potentially active components responsible for observed biological actions.
- Propolis that was first studied could give better results with optimized laboratory protocols
- The pharmacologically active molecules in the propolis are flavonoids and phenolic acids and their esters. These components have multiple effects on bacteria, fungi and viruses.
- Extensive biological screening of traditional propolis will yield better results by using a polarity based solvent system.
- Future in vivo investigations might certify and strengthen the reported in vitro findings.

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