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Antimicrobial Activity of Propolis Extract on Microorganisms

by

Sidra Riaz

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Dedicated to my beloved Mother and memories of my Father.

CERTIFICATE OF APPROVAL

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Abstract

Natural products or material extracted from natural resources as potential drug have been reported to be safer with zero or minimal toxicities. It has been anticipated that approximately over 1/2 of the pharmaceuticals in clinical use these days are derived from natural products. 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. The selected samples of Propolis extract: Propolis 1, Propolis 2 were collected from two different locations. These propolis extracts were screened for antioxidant, antibacterial, antifungal, cytotoxicity, whereas qualitative analysis employed were FT-IR analysis. Manual maceration was the extraction technique. The results of DPPH assay revealed that noteworthy percentage of free radical scavenging was higher observed in Propolis 1 and Propolis 2 with the value of 81 $\pm$ 0.1 and IC50 value is 19.0 and 74 $\pm$ 0.12 and IC50 value is 27.0 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.On the contrary, Propolis 1 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 is higher than Propolis 1 i.e *Fusarium Solani* was 67 $\pm$ 0.1mm and 63.3 $\pm$ 0.1mm respectively. The Minimum percentage of zone of inhibition of Propolis 2 and Propolis 1 i.e. *Aspergilus Fumigants* was 28 $\pm$ 0.01mm and 24 $\pm$ 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 2 with 0.5 $\pm$ 0.1mm (MIC <100) against E.Coli ,0.3 $\pm$ 0.1mm(MIC <100) zone of inhibition against *A.tumefaciens*, and 0.13$\pm$0.1 mm (MIC <100) against *M.Luteusat* 30% concentration. 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 have significant with percentage mortality of 96.66 $\pm$ 0.01 , LC50 value of 180μg/ml and p-value is <0.001, followed by Propolis 1 with percentage mortality of 93.66 $\pm$

0.01, LC50 value of 240μg/ml and p-value is <0.001 at 1000μg/ml concentration respectively.The present research study of tested Propolis extracts confirmed the presence of functional groups that were identified by FT-IR spectroscopy analysis were significant against Carbonyl group(C=O). Our study investigated the natural ethno medicinally significant properties of variety of locally available Propolis of Pakistan, phytochemical evaluation of extracts with their active phytochemical constituent's shows that could be effectively utilized for natural way of treatment. The results have shown that the extracts of this Propolis can be be safely used in pharmacy and other industries as well.

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Abbreviations

%FRSA	Percent Free Radical Scavenging Activity
AAE	Ascorbic Acid Equivalent
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate
FAO	Food and Organization
FCBP	Fungal Culture Bank of Pakistan
FT-IR	Fourier Transform Infrared spectroscopy
IC50	Median Inhibitory Concentration
LC50	Median Lethal Concentration
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
NB	Nutrient Broth
P1, P2	Propolis 1, Propolis2
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
TAC	Total Antioxidant Capacity
ZOI	Zone of Inhibition

Symbols

α	Alpha
β	Beta
γ	Gamma
%	Percentage

Chapter 1

Introduction

Propolis is a term that comes from the Greek word, in which the word: pro sights for “at the entry” and “town/ Community” and polis that represents the use of this natural material in hive protection. Bee glue is a one more term used for propolis [1].The word “propolis” descends as of the Hellenistic Ancient Greek (suburb: bee glue) that starts as of a Greek verb (promalasso) Att., “soften beforehand, make supple by kneading or rubbing” [2]. It was characteristized by Lewis ”the third establishment in production of nectar, a sticky matter which the honey bees use to close the cleft of their hives, honey bee sticks”[3]. It is created by honeybees to defend the hive. Aside from its part in fixing openings, blocking breaks, also smoothing out the inner dividing wall, honey bee stick also use as a disinfectant to avoid bacteriological infection of larvae [4]. Honey bee uses its propolis as antitoxin, which decreases infectious development on hive dividing walls. The shrill coating of propolis gives a resistant covering which confines the departure of water and keeps up consistent humidity inside the hive [4, 5].

Propolis is a resinous material gathered by working drones (Apis mellifera) as of by sucking sap of leaves and flowers of plants. Its organic properties, for example, antibacterial, antiviral, antifungal, among different exercises, have pulled the attraction of scientist’s [6].

Honey and propolis provide useful effect on human well-being. To treat the several diseases especially in folk medicine, it was widely used by human since ancient times. Due to its putrefactive properties, egyptians utilized honey bee paste to deal with their bodies ailments as they certainly understood. Propolis was used as an antipyretic agent. It was used as a mouth antiseptic and an antibacterial and heals up wound by Greek and Roman physicians. Its has been suggested to cutaneous and mucosal wounds suggested for topical therapy [7]. In the 17th Century, Propolis became registered as an authorized drug in London. Between seventeenth and twenty century in Europe, propolis was very famous in Europe due to its antibacterial property. Glue bee is used as a violin varnish in Italy [8]. 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, sore throat, burns and stomach ulcer, propolis was used in the Balkan states [9]. The first experimental work of inclusive of its composition and chemical properties which turned into indexed similarly to chemical abstract was published in 1908 [10].

Owing to resistance to antibiotics by pathogens, current research has been focused towards the usage of old medicine/natural products for handling and control of diseases. Resistance has caused increasing nosocomial infections in pathogen. Propolis is one of natural products that have been verified on pathogens and other in organisms causing community acquired infections. Beside the well-known pathogens, confrontation has also been seemed in opportunistic microorganisms [11].

Propolis is moderately non-poisonous and shows an extensive variety of antimicrobial activities against variety of microorganisms, parasites, and infection [12]. Other organic and pharmacological properties have additionally been investigated for propolis [13]. The therapeutic and antimicrobial properties of propolis have been generally revealed and have a long history [14–16].

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 [17–19]. 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 [20–22].

It is likewise utilized in toothpastes and mouth freshener and to treat gum disease and stomach. It is broadly utilized in beauty care products and in human being nourishments and drinks. It is easily accessible in market as a creams, container, throat capsules, mouthwash 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, pharmaceutical and beauty care product [23].

1.1 Problem Statement

On the contrary, natural products or material extracted from natural resources with as potential drug have been reported to be safer with number or minimal toxicities .It has been anticipated that approximately over 1/2 of the pharmaceuticals in clinical use these days are derived from herbal products. Some natural merchandise-derived tablets which can be an indicator of present day pharmaceutical care consist of quinine, theophylline, penicillin G, Morphine, paclitaxel, digoxin, vincristine, doxorubicin, cyclosporine and vitaminA among many other examples. At present, there is growing need in Propolis treatments because of the aspect consequences related to the artificial drug remedy and Propolis is one in all such natural substance with the drug capacity [24].

1.2 Aims and Objectives

The purpose of this research was to evaluate the "antimicrobial activity of propolis extracts on microorganisms". Propolis became the attention of excessive scientific research during the past 30 years, due to their biotic properties generally expecting its use in human being and Animals medicine, cosmetics and pharmaceutical industry.

1. To explore the natural ethno medicinally significant properties of variety of locally available propolis of Pakistan.
2. Collection of selected Propolis from different local areas of Pakistan.
3. Extraction and phytochemical evaluation of extracts to explore their active phytochemical constituents that could be effectively utilized for natural way of treatment.
4. To screen the Propolis extracts for the exploration of hidden bioactivities of medicinal Significance by employing a set of in vitro bioassays.

Chapter 2

Literature Review

2.1 Historical Point of View

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 [24]. Old Egyptians delineated propolis-production honey bees on vases and also utilized it to treat the numerous sicknesses [25, 26]. In the major century, Cornelius Celsius explained propolis as a treatment for treating injuries, and also for cure of boils [27, 28]. 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. It likewise rarefies, cleanses also douses" [29]. In the Persian compositions propolis is depicted as a treatment against skin swellings, myalgia, also stiffness.

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 comprising bee glue which were used for handling of oral and pharyngeal infections as well as dental caries. In the Georgian original medical piece of writing dated toward c. 1486 Karabadini (Book of Medical Treatment), the writer proposes that propolis is worthy against dental deterioration [30]. 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. Altogether, propolis has been frequently called "Russian penicillin" [31].

2.1.2 Propolis in Initial Modern Era

The interest in propolis came in Europe along though the"Renaissance theory which attracted the interest of people in medicine. The History of Plants (1597), makes the utilization of "the organic compound or substances of poplar tree" for curing purpose [32]. In Seventeenth century, the propolis has been included as an ingredient of drugs for healing purpose in England [33]. On the start of the 19th century propolis was also emphasized as drug by Nicolas Louis Vauquelin, a French apothecary also chemist. In the report prepared toward the Society of Farming Vauquelin describes the propolis or bee mastic that is collected by the bees. It is resinous, yielding, odorant matter of a reddish brown color. "In the mass it is blackish; though it is clear once when in skinny plates. The warmth of the fingers is enough to melt it,however it is additional [34].

FIGURE 2.1: Propolis on honey hive of NARC(As shown in above figure, Propolis is a resinous material of brown in colour, gathered by working dones (Apis mellifera) by sucking sap of leaves and flowers of plants).

2.2 Propolis Bioactive Composition, Properties and Basis

2.2.1 Properties

When heated the propolis, it become soft, gummy, paliable and very sticky. It's a lipophilic in nature, brittle and hard material [35]. 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 [36, 37]. Even transparent propolis has been reported, depends on the resins of origin and it also ranges from yellow-dark brown [38, 39].

2.2.2 Bioactive Proportion

Propolis is acompound 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 [39, 40]. Proportion of different elements exist in propolis, its 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 [40]. During the elaborations of propolis of bees over the resins sugars are supposed to be introduced. Some composites are basic in very propolis trials and that one shows attributed properties.

Various origin propolis comprises of various elements. A few elements are available in various examples from numerous places. A few elements are available in trial from particular plant origin [41]. Because of various climatic condition,its biological activity are fluctuates in distinctive topographical origin trails [42]. 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 2.1. Distinct arrangement is identified with flora particular region and managements of crude material.

2.2.3 Liquefying Degree

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

2.2.4 Solvency

Thinking about the arrangement of propolis, it can't utilize straight forwardly. Propolis exists separated commercially through appropriate solvent. Chloroform, dichloro methane, ethanol, $(CH_3)2CO$, water, ether, and methanolare the best widely utilized extraction solvents. A significant number of the bactericidal segments are dissolvable in H_2O / liquor [43] which must expel all latent solid and

reserve the requires mixture. Its synthesis relies on the geographic district and second one the technique for extraction [44], the dissolvable must be wisely selected [45]. The key diluters utilized for extraction of biochemical compounds and compounds of bioactive remain separated are shown in table 2.2 and 2.3.

2.2.5 Antioxidant Activity of Propolis

To the best of our information, the main reports distributed on the antioxidant activity of Indian propolis are concentrate and its chemical constituent's 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. In addition, it very well may be utilized in protection of different free radical- related disorders. The Galangin indicates comparable activity through that of AEP and EEP and exist in highest activity than pinocembrin. That is because of basic structural changes between these two combinations. Additionally look into is in progress to dissect the constituents of AEP and their antioxidant activity [46].

Its broadened galangin and pinocembrinin 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 was observed in a great degree proficient in combination of Ag and Au nanoparticles [47].

TABLE 2.1: Key plant origin, biochemical compounds and geographical source [48]

Sr. no.	**Plant Origin**	**Geographical Sources**	**Bioactive Compounds**	**Reference**
1	Betula verrucosa Ehrh.	Russia	Polyphenols	[3]

Table 2.1 continued from previous page

Sr. no.	Plant Origin	Geographical Sources	Bioactive Compounds	Reference
2	Predominantly B. dracunculifolia DC.	Brazil	Prenylated p-coumaric	[7]
3	Clusia spp	Cuba, Venezuela	Polyprenylated benzophenones	[89]
4	Unknown	Pacific region (Okinawa, Taiwan)	C-prenylflavanones Furofuran lignans	[90]
5	Unknown	Canary Islands	Furofuran lignans	[15]
6	Unknown	Kenya	Polyphenols	[29, 30]
7	Unknown	Greece and Cyprus	Flavonoids, terpenes	[31]

TABLE 2.2: For the removal of propolis, numerous extraction solvents are used [49].

Water	**Ethanol**	**Methanol**	**Dichloromethane**
Anthocyanins	Terpenoids	Anthocyanins	Terpenoids
Starch	Sterols	Terpenoids	Sterols
Tannis	Alkaloids	Tannis	Alkaloids
Seponins	Tannis	Saponins	Tannis
Polypeptide	Polyphenols	Xnthoxyline	Polyphenols
Terpenoids	Polyacetylene	Tatarol	Polyacetylene
Lectins		Lactones	
		Flavones	
		Polypeptides	
		Polypehnols	
		Lectins	
Ether	**Chloroform**	**Acetone**	
Terpenoids	Terpenoids	Favonols	
Alkaloids	Flavonoids		

Coumarine	Chloroform		
Fatty acids	Terpenoids		
	Flavonoids		
	Chloroform		
	Terpenoids		
	Flavonoids		
	Chloroform		
	Terpenoids		
	Flavonoids		

2.3 Biotic Actions

In propolis, key naturally active component are fluctuate by the usage of various diluters. It's also varying by geographical source and quantity form and is accountable for its various biological Activities [50]. Being there of phenolic esters and flavonoids,it's accountable for that one latent effects with definite reagent.

2.3.1 Antibacterial Action

By agar diffusion method, the antimicrobial activity of propolis composed from Gujarat by agar diffusion method beside *Asparagus nigar, Staphylococcusaureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, and Candida albicans*. Ethanolic extracts of trial (conc. 200 mg/mL) presented lowest action of Gram-negative bacteria (*P. aeruginosa and E. coli*) but great antibacterial action, Gram-positive is *Bacillus subtilis*. Though, *A. Niger* did't shows any action the yeast (*C. albicans*)presented the reasonable zone of inhibition. But, 40% was least the methanolic extracts [51–54].

TABLE 2.3: Geographical Sources, Biochemical compounds and activity in Indian scenario [60].

Sr.no.	Geographic Sources	Activity	Biochemical Compounds	References
1	Karnataka	Antibacterial	Petroleum ether, chloroform, ethanol, methanol, and 40% methanol	58
2	West Bengal	Antioxidant	Ethanol and water	61
3	Gujarat	Antioxidant, antimicrobial	Ethanol, water, petroleum ether, chloroform, ethanol, methanol, and 40% methanol	62
4	Madhya Pradesh	Antimicrobial, hepatoprotective	Ethanol	63
5	Maharashtra	Antimicrobial, antibacterial	Ethanol	64

2.3.2 Antifungal Action

With the presence of flavonoids the fungicidal influence are associated [55]. Andalso influence of propolis on juice fungi spoilage, *C. glabrata, Pichiaohmeri, C. kefyr, C. parapsilosis, C. pelliculosa, Candidafamata* [56]. Within the 40 centuries of strains of, *C. glabrata, C. krusei, C. albicansand and Trichosporonspp*,the propolis is a honey product with greatest antifungal action as verified [57]. Propolis withdrawn the progress of *C. glabrata* (MIC 0.03–7.5 g/mL), *Trichosporon spp.* (MIC 0.1– 0.4 μg/mL),*C. albicans* (MIC 0.2–3.75 μg/mL), and *Rhodotorula sp.* (MIC routinely used antiquaries agents in inhibiting the growth of *Streptococcus* mutant which is a frequent cause of dental caries [58].The concentration improved to 20% and 30%,in ethanolicremoval action was higher through disc diffusion technique. *C. albicans* were not efficient in EEP [59].

TABLE 2.4: The following bacteria used in the recognition of antibacterial activity [64].

Gram-positive	**Gram-Negative**
Bacillus cereus	*Aeromonas hydrophila*
Bacillus subtilis	*Brucella abortus*
Enterococcus spp	*Corynebacterium sp.*
Micrococcus luteus	*Escherichia coli*
Nocardia asteroids	*Helicobacter pylori*

2.3.3 Vaginal Usage

By the Brazilian propolis, micro particles (PMs) are articulated [61] and [62] and isolates of significance in the Vulvovaginal Candidiasis (VVC) to test pastime of the propolis extract (PE) against clinical yeast *C. Albicans and 31 non-C.Albicans (C. Glabrata, C. Tropicalis, C. Guilliermondii, and C. Parapsilosis*).Furthermore, for the management of VVC also been tested by using the main antifungal pills. Amphotericin B. Non-*C. Albicans* isolates presented better resistance and dose-based susceptibility for the azolic pills than *C. Albicans*. Though, for Amphotericin B, all have been touchy or dose-established. Through the PE and PMs, with small variant, independent of the species of yeast had been inhibited. The overall outcomes provided vital records for the ability software of PMs within the remedy of VVC and the possible prevention of the incidence of latest indicative incidents [63].

2.3.4 Anti-Protozoal Action

Afterward incubation in the existence of various concentrations of propolis, antiprotozoa action is assessed by an invitro growth inhibitory influence on the culture of parasites [65]. The diseases caused in humans and animals such as toxoplasmosis, Chagas disease, leishmaniasis,giardiasis, malaria and trichomoniasisby the influence of European propolis on protozoalstated by numerous journals. Trichomonas vaginalis, Toxoplasma gondii, Giardia lamblia, Leishmania donovani,

and Trypanosoma cruzi [66]. Against the G. duodenalis, anti protozoan activity of EEP was stated [67].

2.3.5 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 [68]. 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 [69].Though the caffeic acid,An antimetastatic activity,phenethyl esters (CAPE) from poplar propolis and Artepillin C from Baccharis propolis have been recognized as the greatest effective antitumor agent in various polyphenols [70], [71]. 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, zero.7, and 1.Zero mL)[72]. The suggest micronucleus quotes had been 1.4770.38 - 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)[73]. In peripheral human being lymphocytes in vitro are acquaintance to various concentrations of propolis cannot produce a cancer-causinginfluence. Though, it showed that propolis might have a cancer-causing influence in high concentrations by increasing micronucleus (MN) rates [74].

2.3.6 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 [75]. The action of propolis has been looked into by Almeida and Menezes. NADPH-oxidase ornithine decarboxylase, Myeloperoxidase movement, tirosine-proteinkinase, and hyaluronidase from guinea pig pole cell shave inhibitory properties of propolis. Through the existence of flavonoids dynamicand cinnamic acid byproducts the anti-inflammatory action can be described [76]. The former comprises of naringenin,quercetin, andacacetin; the latercontainscaffeic acid (CA) and caffeic acid phenyl ester (CAPE) [76]. Previous incorporates,naringenin,quercetin, andacacetin the last includes caffeic corrosive (CA) and caffeic corrosive phenyl ester (CAPE) [74].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 [75]. 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 [76].

2.3.7 Hepatoprotective Action

Defensive capability of a propolis changed into assessed alongside mercury-incited oxidative pressure then most cancers prevention agent enzymatic adjustment in liver of mice.By using the increasing lipid peroxidation & oxidized glutathione level and introduction to a mercuric chloride incited oxidative fear alongside corresponding abatement in glutathione and extraordinary most cancers prevention agent proteins. Mercury inebriation strayed the movement of marker liver compound in blood. Conjoint remedy of propolis repressed lipid peroxidation and oxidized glutathione level even though improved stage of glutathione. Action

of cancer prevention marketer's catalysts, that is, catalase, superoxide dismutase, glutathione S-transferase, and glucose 6-phosphate dehydrogenase, became moreover reestablished correspondingly closer after propolis organizationto control. Arrival of serum transaminases, lactate dehydrogenase, soluble phosphatase, and γ-glutamyltranspeptidase become basically reestablished closer to control after propolis remedy. Results propose that propolis increases the cancer prevention agent protect in opposition to mercury-actuated poisonous first-class and gives proof that it has remedial ability as hepatoprotective specialist.

2.3.8 Anti-Diabetic Action

The impact of ethanolic listen of propolis against trial diabetes mellitus-related adjustments becomes inspected. Diabetes becomes incited tentatively in rats by using i.P. Infusion of streptozotocin (STZ) in measurements of 60 mg/kg between for three innovative days. Blood urea nitrogen (BNU), creatinine, glucose, lipid profile, malondialdehyde (MDA), and urinary egg whites have been predicted. Superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and MDA were predicted inside the renal tissue. The consequences indicated diminished frame weight and increased kidney weight in diabetic creatures [77]. Contrasted with the manage everyday rats, diabetic rats had higher blood glucose, BNU, creatinine, add up to cholesterol, triglycerides, low-thickness lipoprotein-ldl cholesterol (LDL-C), MDA and 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, 2 hundred, and three hundred mg/kg bwt better the frame and kidney weights, serum glucose, lipid profile, MDA, and renal capacity exams. Renal GSH, SOD, and CAT had been altogether increased whilst MDA turned into significantly decreased [78].These results may additionally suggest a strong cancer prevention agent impact of propolis which can enhance oxidative stress and delay the occasion of diabetic nephropathy in diabetes mellitus [79].

2.3.9 Immunomodulatory Action

The immunomodulatory interest of a water-solvent subsidiary (WSD) of common propolis was tested. The oral and parenteral business enterprise of the WSD improved the survival price and the suggest survival time in exploratory bacterial (*Klebsiella pneumoniae*, *Staphylococcus aureus*) and parasitic (*Candida albicans*) contaminations in mice [80]. 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 non specific host resistance with the aid of macrophage initiation [81].

2.3.10 Dental Action

The antimicrobial motion of 5 propolis tests accrued from 4 specific locales in Turkey and from Brazil in against to 9 anaerobic (*Peptostreptococcus anaerobius, Peptostreptococcus micros, Prevotell aoralis, Prevotell amelaninogenica, Porphyro monasgingivalis, Fusobacter iumnucleatum, Veillon ellaparvula, Lactobacillus acidophilus, and Actino mycesnaeslundii*) lines 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 weakening method [82]. All traces were defenseless and MIC esteems ran from four to 512 mg/mL for propolis movement. Propolis from Kazan-Ankara indicated pleasant MIC esteems to the pondered microorganisms. MBC estimations of KazanAnkara EEP exams ran from eight to 512 mg/mL [83]. Demise become visible inside four h of brooding for *Peptostreptococcus anaerobius* and micros and *Lactobacillus acidophilus* and *Actino mycesnaeslundii*, while being eight h for *Prevotellaoralis, Prevotell amelaninogenica*, and *Porphyro monasgingivalis*, 12 h for *Fusobacterium nucleatum*, and 16 h for *Veillonell aparvula*. It become validated

that propolis tests had been more compelling against Gram-advantageous anaerobic microscopic organisms than Gram negative ones [84, 85]. Propolis is applied in oral cavity sicknesses because it carries flavonoids, for instance, pinobanksin, quercetin, naringenin, galangine, chrysin, and fragrant acids, as an instance, caffeic corrosive controlled by using GC-MS exam [86, 87].

Chapter 3

Materials and Methods

Following research work was carried out in biological laboratory of Department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad.

3.1 Materials

Material utilized for the research work is given below 3.1:

Table 3.1: Material Utilized for Research Work

Chemicals	**Company Name**
Methanol	Sigma-Aldrich
Distilled water	-
DPPH reagent(2,2-diphenyl-1-picrylhydrazyl)	-
Ascorbic Acid	-
Terbinafine	-
Streptomycin	-
Nutrient Agar	-
Sabouraud Dextrose Agar(SDA)	-
Brine Shrimps egg	-

Table 3.1 continued from previous page

Chemicals	**Company Name**
Sea salt	-
Consumables	
Petri plates	
Test tubes	
Vials	
Micropipette	
Cotton plugs	
Cotton swabs	
Aluminum Foil	
Falcon tubes 15ml, 50ml	
Eppendorf tubes	
Beaker 100ml, 500ml, 1000ml	
Test tubes racks	
Discs	
Para film or masking tape	
Forceps	
Microorganisms Used	
Bacillus subtilis	
AT-10	Aspergillus fumigatus
Staphylococcus aureus	Aspergillus niger
Enterobacter aerogenes	Mucor Species
Micrococcus luteus	Fusarium solani

3.2 Methods

3.2.1 Research Methodology Outlines

Figure 3.1 shows the detail outlines of our research methodology.

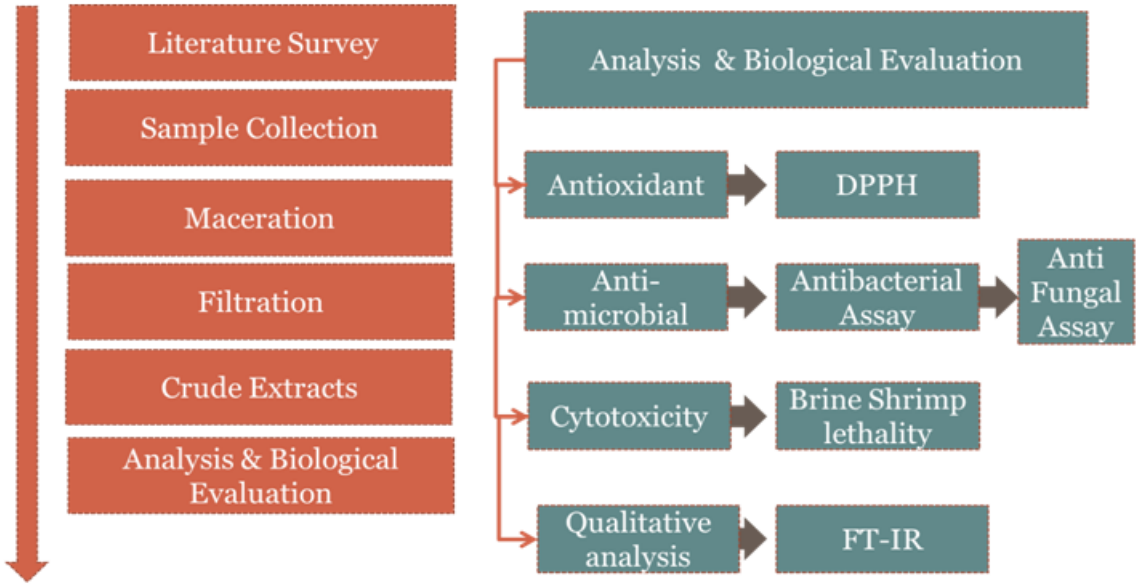

FIGURE 3.1: Detail outlines of our research methodology

FIGURE 3.2: Sample (Propolis1) Collection from NARC (National Agriculture Research Centre)

3.2.2 Samples Collection

In the recent study, two different propolis samples were collected from different areas of Pakistan. One of the Propolis sample was collected from the hives of Honey Research Institute of NARC (National Agriculture Research Centre) and was tagged as propolis1. And other Propolis sample was collected from Dama jungle Wang awal, Rajanpur and tagged as propolis 2. All the propolis samples were in dried form, properly kept at refrigerator at 4°C.

3.2.3 Extraction

Extraction technique employed was manual maceration. Accurately weighed (10gm) of propolis samples were crushed into small pieces and extraction was done in 70% Methanol in 100ml. And it was left overnight at room temperature. Second day, the suspension was filtered and the resulting extract was kept in refrigerator at 4°C [86].

3.3 Biological Evaluation of Propolis Extract

3.3.1 Antioxidant Assays

Antioxidant capacity of Propolis samples was determined by using DPPH method (2, 2-diphenyl-picryl-hydraxyl-hydrate) that was described by Khan et al. (2015) [87].

3.3.1.1 Sample Preparation

Stock was prepared by adding distilled water in the Propolis extract different dilutions were used for antioxidant assays (10, 20, 30 μM).

3.3.1.2 Preparation of DPPH Solution (Free Radical Scavenging Assay; FRSA)

12 mg of DPPH was added in 100 ml methanol in order to freshly prepare DPPH solution. Preparation of ascorbic acid solution was done by adding 1 ml of DMSO to 1 mg of ascorbic acid. Crude test extracts were weighed and a stock solution of 4 mg/ml of each test extract was prepared in Methanol.

3.3.1.3 Procedure

Free radical extinguishing capability of extracts or samples is assessed by DPPH reagent based assay. A change in absorbance values is detected because antioxidants in test samples cause production of hydrazine which renders 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 (200ul) 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°C in a pitch dark surrounding and measured absorbance at 517 nm with the help of spectrophotometer reader and % scavenging activity of each propolis sample was find out by the given formula:

$$\%Scavenging = (1 - \frac{Abs}{Abc})100 \tag{3.1}$$

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.

3.3.2 Antimicrobial Assays

Antimicrobial Assays: There are two kinds of antimicrobial assays were executed to evaluate the biological activity of propolis extract.

- Antibacterial assays
- Antifungal assays

3.3.2.1 Antibacterial Assays

Five strains of bacteria were used for antibacterial assessment. Antibacterial properties of propolis extract was analyzed by means of disc diffusion method as described by Khan et al.,[88].

3.3.2.2 Bacterial Strains Used

- *Bacillus subtilus*
- *AT-10*
- *Staphylococcus aureus*
- *Micrococcus luteus*

3.3.2.3 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.

3.3.2.4 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°C for 24 hrs.

3.3.2.5 Media for Bacterial Growth

Nutrient Agar was used for bacterial growth in petri plates. Add 28g of Nutrient agar in 1 liter of distilled water. The composition of Nutrient Agar is as under:

1. Peptone 5g/500ml
2. Yeast Extract 3g/500ml
3. Agar 15g/500ml
4. Sodium Chloride 8g/500ml
5. Distilled water 1 liter

3.3.2.6 Procedure

By taking 50 μl aliquot from 24 hrs refreshed bacterial cultures was used to prepare lawn on Nutrient Agar petri plates. Two 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. One of positive controls (Streptomycin)were also infused on discs and placed on plates. And other one of negative control that was distilled water. At 37°C for 24 hrs incubation was done. 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.

3.3.3 Antifungal Assay

For determining the antifungal activity of propolis extract, Tube dilution method was used [89, 90].

3.3.3.1 Preparation of Sample

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

3.3.3.2 Inoculum Preparation

Spores of fungal strains were harvested from stock cultures on sterile SDA plates. At 28°C, incubation of plates was done for 7 days.

3.3.3.3 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

3.3.3.4 Use of Fungal Strains

Four strains of fungus were used for the antifungal assays.

- *Aspergillus flavus*
- *Aspergillus fumigatus*
- *Aspergillus niger*
- *Mucor Species*

3.3.3.5 Procedure

Antifungal assay was performed as previously illustrated by [89, 90]. Mark test tubes at 10cm. Add (5ml) having sterile sabouraud dextrose agar were swabbed

with 100μl refreshed inoculum and make slants. Cover test-tubes with cotton plugs. Place the Positive standard (Terbinfine) and Negative standard on test tubes. At 37°C for 4 days incubation was done. The fungal growth on test tubes was measured by vernier Caliper. The assay was run as triplicate analysis. Following formula was used to calculate the percentage growth inhibition:

$$PercentageViability = \frac{Negativecontrol - test}{NegativeControl} * 100 \tag{3.2}$$

3.3.4 Cytotoxicity Assays

Brine shrimps cytotoxic assay was performed to determine the level of toxicity of propolis extract as reported earlier [91].

3.3.4.1 Preparation of Samples

The 10 mg/ml stock solutions of all propolis extracts were prepared in100ml Methanol. Standard drug doxorubicin stock solution was prepared as 4 mg/ml.

3.3.4.2 Sea Salt Preparation

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

3.3.4.3 Hatching of Eggs

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

3.3.4.4 Procedure

The preliminary cytotoxicity of crude extracts against brine shrimps (Artenia salina) larvae was determined by 24 hrs lethality test as described previously by

[91]. Artenia salina eggs were hatched in specially designed bi-compartment perforated tank that was filled with simulated sea water. The compartment containing eggs was completely covered with aluminium foil while other was lightened with a light source. The tank was incubated at 30-32°C for 24-48 hrs. After specified incubation period, the eggs were hatched and nauplii started moving towards the lightened compartment of the tank through small perforations. The hatched nauplii were then collected with Pasteur pipette and placed in beaker containing sea water. Two-fold serial dilution of test extracts was made up to the final concentrations (1000, 500, 250 μM). 15 mature nauplii were transferred and 150 μl of sea water was added to each vial. After incubating at 25°C 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:

$$\%mortality = \frac{no.of deadshrimps}{totalno.of shrimps} * 100 \tag{3.3}$$

3.3.5 Qualitative Analysis

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

3.3.5.1 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.

Procedure:

All the propolis extracts were analyzed by FT-IR Qualitative Analysis (KBr pellet method) by using Fourier Transform Infrared Spectrometer (Bruker-Tensor 27) instrument under the following appropriate conditions:

- Instrument: Bruker-Tensor 27; FT-IR.
- Spectral range: 515 cm-1 - 4000 cm-1.
- Resolution: 4 cm-1.

The acquired spectra for the products were examined and construed for particular infrared absorption frequencies with a table to characterize the functional groups for organic and carbonyl compounds. Each functional group has different absorption frequencies and Omnic software 8.2 was used for the interpretation of FT-IR spectra [91–93].

Chapter 4

Results and Discussion

4.1 Biological Evaluation

4.1.1 Antioxidant Potential (DPPH assays)

Stability and accessibility inside the cells make DPPH free radical a perfect criterion to check scavenging potentiality and consequently, and also antioxidant ability in test extracts. DPPH reagent is of dark purple color and it has the capability to gain an electron from donor antioxidants resulting in change of color from dark purplish to light purple up to light yellow. This decolorization is owing to the presence of antioxidants in propolis extracts which can be quantified by computing changes in absorbance values at 517 nm by spectrophotometer [94]. The potential free radical scavenging activity of all the Propolis extracts was determined by DPPH assay (Figure 4.1). The results of DPPH assay revealed that noteworthy percentage of free radical scavenging was higher observed in Propolis 2 than propolis 1 with the value of 81 $\pm$ 0.1 and IC50 value is 19.0 and 74 $\pm$ 0.12 and IC50 value is 27.0 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. The % scavenging of all the Propolis samples were as follows [Table 4.1]. The IC50 values of propolis samples were calculated by using Graph pad

prism 5 software. The IC50 value of Propolis 1 and Propolis 2 were 27.0 and 19.0 respectively 4.1. The free radical scavenging activity ofall the active samples in terms of % scavenging and IC50 followed in the order:

$$(NARC)Propolis1 < Propolis2(forestofRajanpur) \tag{4.1}$$

In vitro characterization of propolis extracts have been found out on the basis of scavenging of stable free radicals by using DPPH assay. 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 figure4.2, which might be ascribed to the different functional groups present in propolis2 extract as confirmed by FT-IR analysis. And also confirmed from previous findings and the reason might be some factor as geographical regions, climate conditions flora,cultivating and harvesting time periods, moisture and storage. Our results are in close agreement with the previously reported work where maximum free radical scavenging activity was observed in propolis [95].

FIGURE 4.1: DPPH free radical scavenging activity of selected propolis extracts by spectrophotometric method.

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 20 30	18.10 ± 0.1 42 ± 0.5 74 ± 0.1	27
Propolis 2	10 20 30	21 ± 0.3 54 ± 0.54 81 ± 0.1	19
Positive control	10 20 30	20 ± 0.33 45 ± 0.55 66 ± 0.1	16
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**	**Significance**
Interaction	199.4	2	99.7	5.310	<0.0001	Yes
Types of propolis	439.1	1	439.1	23.38	<0.0001	Yes
Concentration	100.30	2	5017	267.27	<0.0001	Yes
Residual	225.3	12	18.78			

4.2 Antimicrobial Potential

4.2.1 Antibacterial Activity

Anti-bacterial 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 table4.3. Propolis 1 showed maximum activity against *M.luteusi*.e. (0.1 ± 0.1mm) and *A.tumefaciens* (0.2 ± 0.1mm). The weakest activity of propolis 1 was observed against *E.coli*. i.e. 0.013 ± 0.01mm respectively table 4.3. In table 4.3, 0,-, = shows No activity, Propolis 2 showed maximum activity against

A.tumefaciens (0.3 ± 0.1 mm) and *E.coli* (0.5 ± 0.1mm). The weakest activity of propolis 2 was observed against *M.luteus* (0.13 ± 0.1) table 4.3.

The results of our study are in harmony with Hendi *et al.*, (2011) manifested the inhibitory effect of propolis against *K. pneumonia* and *E.coli* [96]. Hendi *et al.*, (2011)revealed that phenolic compounds of propolis can be contributed to the antibacterial activity, causing the release of intracellular membrane components as amino acids, proteins, pentose and phosphates leading to the membrane perturbation and permeability and also inhibited lipid peroxidation [96]. 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 antibiotics Streptomycin were used as positive control and revealed minimal antibacterial potential against bacterial strains as shown in table 4.3. Distilled water was used as negative control showed no antibacterial activity that proved its harmless effects on the tested bacterial strains. The results of our research work confirmed the presence of different bioactive compounds in our tested extracts that might contributed to antibacterial activity, as verified by FT-IR techniques.

TABLE 4.3: % Inhibition against bacterial strains of selected propolis extracts.

Samples at different	**Antibacterial Assay**								
Concentration (ppm)	**Diameter of zone of inhibition in mm (Mean ± SD) * (MIC: μg = ml)**								
	M.luteus	MIC	*E.aerogenes*	MIC	*A.tumefaciens*	MIC	*B.subtilis*	MIC	*E.coli*
Propolis1									
10%	0.1 ± 0.1	<100	0	<100	0.1 ± 0.01	<100	0.006 ± 0.01	<100	0.01 ± 0.01
20%	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%	0.1 ± 0.1	<100	0.13 ± 0.01	<100	0.2 ± 0.1	<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	-		-		-		-		-
Propolis2									
10%	0.06 ± 0.01	<100	0.01 ± 0.01	<100	0.1 ± 0.1	<100	0.006 ± 0.01	<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%	0.13 ± 0.1	<100	0.1 ± 0.1	<100	0.3 ± 0.1	<100	0.17 ± 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	-		-		-		-		-

M.luteus;Micrococcus luteus, A.Tumefacicens; Agrobacterium tumefaciens, B.subtilius; Bacillus subtilis, E.coli; Escherchia Coli, MIC;Minimium inhibitory Concentration.

4.2.2 Antifungal Activity

By employing agar dilution method, crude extracts of all the propolis were investigated for their antifungal potential against fungal strains. 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 maximum percentage of zone of inhibition of fungal strains of Propolis 2 is higher than Propolis 1 i.e *Fusarium solani* was 67 $\pm$ 0.1mm and 63.3 $\pm$ 0.1mm respectively.The Minimum percentage of zone of inhibition of Propolis 2 and Propolis 1 i.e. *Aspergilus fumigants* was 28 $\pm$ 0.01mm and 24 $\pm$ 0.01mm respectively,the assay was run as triplicate analysis [96]. The percentage of inhibition against Fungal strains of selected propolis extracts:

$$(NARC)Propolis1 < Propolis2(forestofRajanpur) \tag{4.2}$$

TABLE 4.4: % Inhibition against Fungal strains of selected propolis extracts.

S.No.	***Fusarium solani***	***Aspergilus mucor***	***Aspergilus fumigants***	***Aspergilus niger***
Propolis 1	63.3±0.1	44±0.1	24±0.01	31±0.01
Propolis 2	67±0.1	48±0.4	28±0.01	38±0.01
Positive Control	100	100	100	100
Negative control	0	0	0	0

4.3 Cytotoxicity Potential

4.3.1 Brine Shrimp Lethality Assays

Earliest cytotoxicity of the Propolis was assessed against Arternia salina nauplii (brine shrimp larvae) and the obtained results were analyzed to determine the

lethality profile of the selected propolis by employing the brine shrimp Lethality test figure 4.2.

FIGURE 4.2: Brine Shrimp lethality assay.(a) *Artemia saline* eggs in bicompartment perforated tank; (b) Hatched nauplii in vials containing sea water.

This assay is based on the ability of samples to kill the brine shrimp larvae. This assay has been considered as an efficacious probe for the bioactivities of different plants extracts [97]. Overall crude extracts exhibited significant mortality and results were depicted in table 4.6. In this research, three different concentration (1000, 500,250) 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 96.66 $\pm$ 0.01 , LC50 value of 180 μg/ml and p-value is < 0.001, followed by propolis 1 with percentage mortality of 93.66 $\pm$ 0.01, LC50 value of 240 μ g/ml and p-value is < 0.001 at 1000 μg/ml concentration respectively table 4.4. The cytotoxic potential of the Propolis extracts arranged in the following manner:

$$(NARC)Propolis1 < Propolis2(forestofRajanpur) \tag{4.3}$$

It was observed that the viability of shrimps were considerably decreased as the higher concentration and had more mortality rate than lower concentrations of Propolis extract table 4.5. It is commonly inferred that brine shrimps or *Arternia salina* larvae and carcinoma cells of mammals behave in the same manner in many aspects that is why cytotoxic effects of undertaken test extracts might

TABLE 4.5: Brine shrimps lethality potential of selected Propolis extracts.

Cytotoxicity Potential			
Samples Names	**Concentration(μgml)**	**%Mortality**	LC50(μg/ml)
Propolis 1	1000	93.66±0.01	240
	500	60±0.01	
	250	53±0.01	
Propolis 2	1000	97±0.01	180
	500	90±0.01	
	250	83±0.01	

become potential candidates for antitumor and anticancer activities; possible biological activities of test extracts against malarial parasites, pests, tumors and harmful microbes [98]. The activity of samples were based on concentration dependent manner and as there was decrease in concentration of samples, the percent (%) mortality rate also de-creased confirmed the prior studies by using the brine shrimps larvae as a test model figure 4.4 [99].

TABLE 4.6: Analysis of Variance for Factors Effecting the Viability of Brine Shrimps.

Source of Variation	**Sum of Squares**	**Df**	**Mean Square**	**F-Value**	**P-Value**	**Significance**
Interaction	1339	2	223.2	21.02	<0.001	Yes
Types of propolis	27620	2	9206	866.8	<0.001	Yes
Concentration	3197	1	1599	150.5	<0.001	Yes
Residual	254.9	13	10.62			

4.4 Qualitative Analysis

4.4.1 Determination of Functional Groups using FT-IR Spectroscopy

For the identification of functional groups, the most common widely used technique is FT-IR spectroscopy. FT-IR spectroscopy is speedy, versatile and responsive technique that has been utilized for illustrating the structure and physiochemical properties of investigated material [100]. In this technique, functional groups can be detected depending on the extract composition and also on the solvent polarity. For the characterization of crude extracts of Propolis, FT-IR spectroscopy was conducted figure 4.3. The present research study confirmed the presence of functional groups that were identified by FT-IR spectroscopy analysis. Figures and table presented the infra-red spectrum of each Propolis and characteristic bands were observed ranging from 4000 cm-1 to 515 cm-1 in all Propolis samples spectrum.

TABLE 4.7: FT-IR analysis of Propolis extracts; Propolis 1 (P1) and Propolis2 (P2)

Sr.No.	**Frequency of band (cm-1)**	**Experimental Frequencies of Propolis(cm-1)**	**Bond**	**Functional groups**
1	3500-3200	3311.12 P1 3311.12P1 3313.61 P2 3313.61P2	O-H Stretch, H-bonded	Alcohols, Phenols
2	3000-2850	2943.80P1 2943.80P1 2943.67P2 2943.67P2	C-H Stretch	Alkanes

Table 4.7 continued from previous page

Sr.No.	**Frequency of band (cm-1)**	**Experimental Frequencies of Propolis(cm-1)**	**Bond**	**Functional groups**
3	3300-2500	2833.51P1 2833.51P1 2831.63P2 2831.63P2	O-H Stretch	Carboxylic acid
4	1740-1720	1639.10P1 1639.10P1	C=Stretch	Aldehydes, Saturated
				aliphatic
5	1760-1690	P1,P2	C=O Stretch	Carboxylic acid
6	1760-1665	P1,P2	C=OStretch	Carbonyls (general)
7	1710-1665	P1,P2	C=O Stretch	Unsaturated aldehydes,
				Ketones
8	1680-1640	P1,P2	-C=C-Stretch	Alkenes
9	1650-1580	P1,P2	N-H Bend	1 amines
10	1550-1475	P1,P2	N-O Asymmetric stretch	Nitro compounds
11	1500-1400	1449.68P1 1449.68P1 1449.15P2 1449.15P2	C – C Stretch	Aromatics
12	1370-1350	1269.84P1 1269.84P2	C –H Rock	Alkanes
13	1335-1250	1115.11P2 1166.61P1	C- N Stretch	Aromatic amines

Table 4.7 continued from previous page

Sr.No.	Frequency of band (cm-1)	Experimental Frequencies of Propolis(cm-1)	Bond	Functional groups
14	1300-1150	1166.61P1 1115.11P2 1115.1P2	C-H Wag	Alkyl halides
15	1250-1020	1114.37P1 1114.37P1 1115.11P21 115.11P2	C-N stretch	Aliphatic amines
16	1320-1000	1019.71P1 1019.71P1 1021.61P2 1021.62P2	C-O Stretch	Alcohols, Carboxylic acids, Esters, Ethers
17	1000-650	604.19P1 604.19P1 615.06P2 600.05P2	=C-H Bend	Alkenes
18	850-550	591.01, 577.98, 562.92P1 578.67, 569.16, 531.13P2	C-Cl Stretch	Alkyl halides
19	690-515	543.08, 517.03, 527.35P1 543.47, 522.10P2	C-Br Stretch	Alkyl halides

The results summarized in the table 4.7 show the presence of highest absorption band in the region of 3500-3200 cm-1 in all the propolis. This band is caused by the presence of alcohol and phenolic groups and/or the H-bonded O-H stretch in hydration water. It means propolis possessed hygroscopic characteristic and exhibit hydrophilic nature [100]. Below 3000 cm-1, the saturated hydrocarbons C-H stretch occurs. The strong bands appear at 850 cm-1 to 550cm-1 and 690cm-1 to 515cm-1 in all the proporlis indicated the stretching of C-Cl and C-Br in Alkyl halides [101]. Another strong absorption band at 2849 cm-1 was also observed due to O-H stretching, indicated the presence of carboxylic acid group in all theproporlis. Carbonyl group is the significant functional group consist of C=O. In the spectra, carbonyl compounds are the strongest bands lie in the region of 1760 cm-1, 1665 cm-1 indicated the presence of aldehydes, saturated aliphatics, Carboxylic acid, α, β unsaturated aldehydes, Ketones and Carbonyls (general). For the functionality of double bond, conjugation plays significant role in the observing carbonyl frequency. The band between 1500 cm-1 to 1400 cm-1 in propolis indicated the presence of aromatic compounds that contributed to antioxidant and other biological activities of propolis, supports the confirmation of our results table 4.5. The another strongestband was also observed at 1030 cm-1 confirmed the presence of esters, carboxylic acid, ether and alcoholic compounds in our all test extracts that also proved their strong aroma, taste and these compounds play significant roles in bio activities of propolis. Many small peaks were observed between 1370 cm-1 1020 cm-1 and 970 cm-1, 522 cm-1, confirmed the presence of many functional groups. 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) [102]. 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.

4.4.2 Biochemical Analysis of Samples via FT-IR

The significant spectral range present between 3500-515 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 compositions [103]. 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.

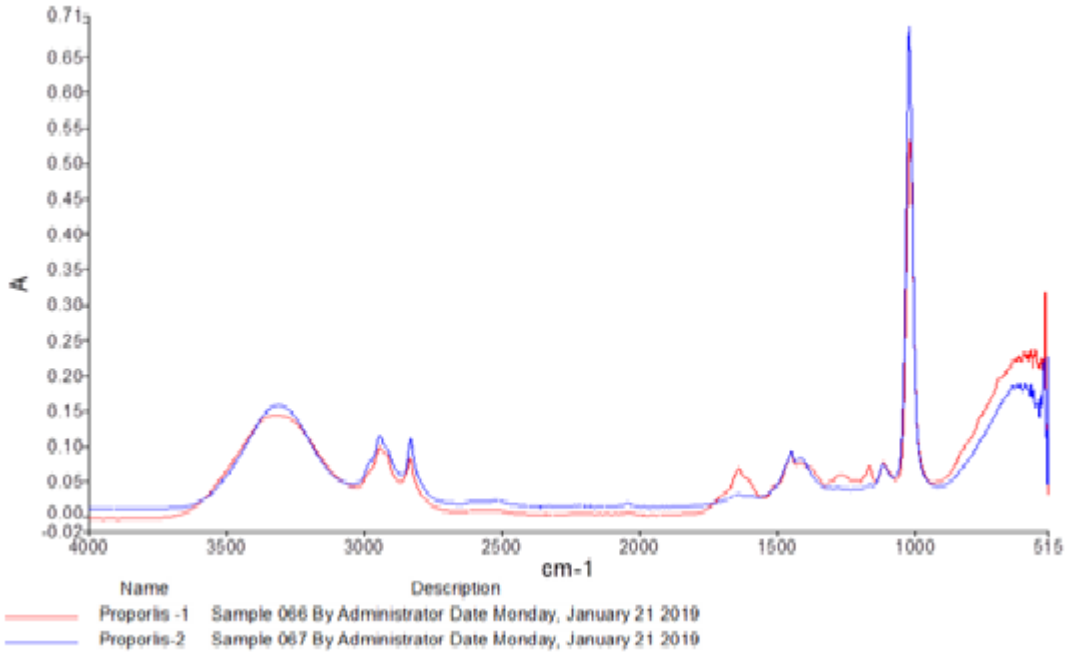

FIGURE 4.3: (a) Absorption Spectrum View of Propolis 1 and 2

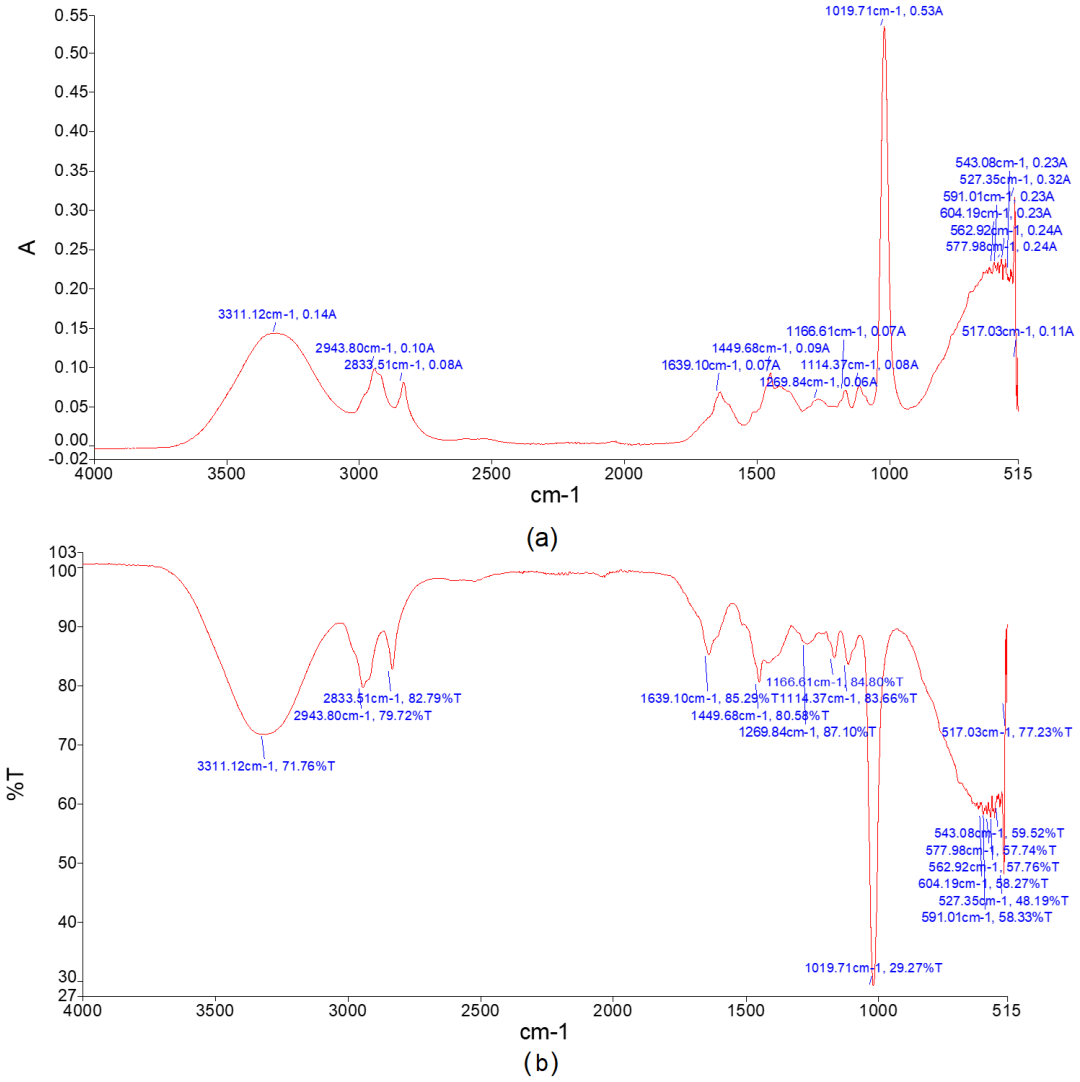

FIGURE 4.4: (a) Absorption and (b) Transmission spectra of Propolis 1 . FT-IR spectrum of propolis 1 showing significant functions groups for phytochemical, antioxidant, antimicrobial, cytotoxicity activities.

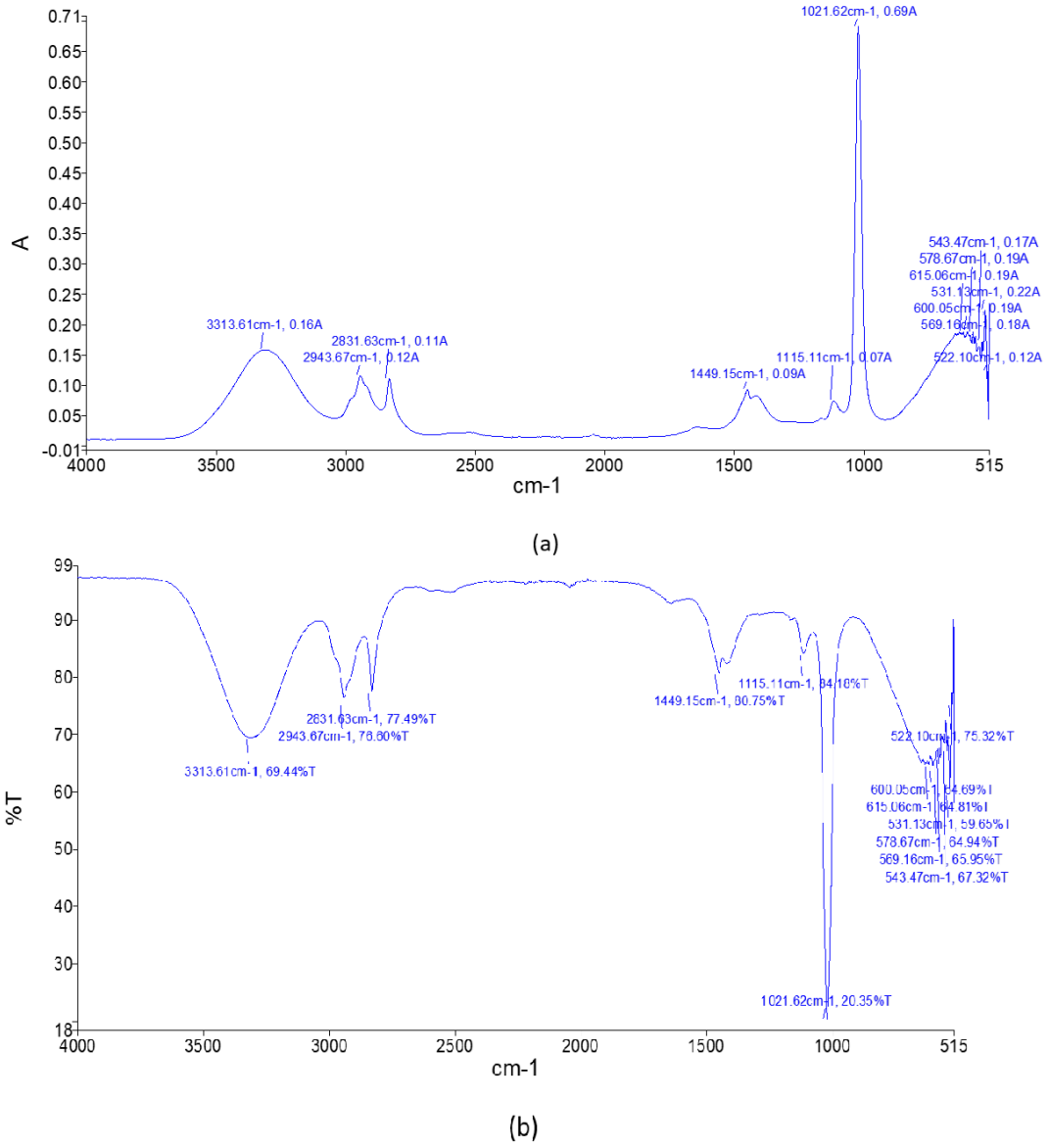

FIGURE 4.5: (a) Absorption and (b) Transmission spectra of Propolis 2 . FT-IR spectrum of propolis 2 showing significant functional groups for phytochemical, antioxidant, antimicrobial, cytotoxicity activities.

Chapter 5

Conclusion and Future Prospects

5.1 Conclusion

Current research has been focused towards the usage of old medicine/natural products for handling and control of diseases. Propolis is a natural product that is being investigated against pathogens and also organisms causing community acquired infections. Beside the well-known pathogens, resistance has appeared in opportunistic microorganisms. Antimicrobial resistance results in increased illness, deaths, and health-care costs, highlighting the need for novel antimicrobial agents .Propolis is widely utilized in folk medicine, and various examinations have demonstrated that Propolis is antibacterial, antiviral and antifungal properties. Propolis is non-poisonous and shows an extensive variety of antimicrobial activity against variety of microorganisms.

In conclusion, assaying of maximum antioxidant aptitude narrated as ascorbic acid equivalent was also computed highest most in propolis 2 extract whereas propolis 1 extract showed less antioxidant Potential. In antibacterial assay, all of the extracts of propolis were active against five bacterial strains tested that confirm their use and efficacy against various infections. Among them, remarkable activity was shown against *M.luteus, A.tumefaciens, B.subtilis* by Propolis 1 and Propolis 2 extracts however; modest activity was observed against *A.tumefaciens and E.coli*

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 against the fungal strains tested in our study.

Cytotoxicity profile established using brine shrimp lethality assay confirmed the highest efficacy of Propolis 2 extracts that may proposed their utilization as anti-cancer and anti-mutagenic agents while minimum activity was observed in Propolis 1.

All the tested propolis extracts confirmed the presence of significant functional groups that were identified by FT-IR spectroscopy analysis. Results of our detailed screening led us to the conclusion that the probing of Propolis has unveiled the additional benefits of these Propolis and also exhibited promising perspective for the discovery of new bioactive molecules. The results have shown that the extracts of this Propolis could be safely used in pharmacy and other industries as well. So, more investment and research is needed for the screening of bioactive compounds of traditional Propolis which could serve as an effective means for therapies.

5.2 Future Prospects

- By employing polarity based solvent system, extensive biological screening of traditional propolis will provide better results.
- Propolis which was studied first time might give better results by optimized lab protocols.
- 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.
- Future *in vivo* investigations might certify and strengthen the reported *in vitro* findings.

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