Evaluation of Bactericidal and Fungicidal activity of Indian Propolis

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Abstract

From the plagues of biblical times to the recent COVID-19 pandemic, infectious diseases have played an undeniably key role in human health. A disease that occurs through the invasion of a host by a foreign agent whose behavior harms or impairs the physiological functioning of the host's system is pathologically termed an infectious disease. Several factors have been implicated in the etiology of infectious diseases, including increasing population, poverty, malnutrition, social practices, lack of awareness, increased domestic and global connectivity, and illiteracy. Above all, the frequency in the prevalence of diseases due to pathogenic microorganisms has increased alarmingly due to the development of resistance to available drugs, and the present scenario necessitates the incessant search for new classes of antimicrobial agents, preferably from natural resources. Propolis stands for an icon among various cultures owing to the utility that it bestows which renders immense benefit to mankind. The present study was carried out to evaluate the possible antibacterial and antifungal efficacy of an ethanolic extract of Indian propolis by well diffusion (bacteria) and disc diffusion (fungi) methods. The Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC) were also determined by established procedures. The bacterial and fungal strains were chosen based on their clinical significance. Totally five Gram-positive, five Gram-negative, and eight fungi were screened using graded concentrations of propolis extract. The results obtained on the zone of inhibition and visible growth lead to the conclusion that the Indian propolis extract possesses a broad spectrum of antibacterial and antifungal activity. The present study also provides evidence for the use of propolis in traditional medicine for the treatment of microbial infections and forms the basis for the isolation of bioactive compounds with significant antimicrobial activity from the Indian propolis for food preservation and therapeutic applications.

Keywords: Indian propolis, Antibacterial activity, Antifungal activity, Minimum Bactericidal Concentration, Minimum Fungicidal Concentration.

Introduction

The majority of microorganisms such as soil born, intestinal, industrial and commercial application oriented elicit critical contributions to the welfare of the world's inhabitants by aiding to maintain the balance of living things and chemicals in the environment. With the advent of the germ theory of diseases, the pivotal role of microbes in causing infectious diseases has been established, setting the stage for the beginning of the 'modern antibiotic era'. Though only a minority of microorganisms is pathogenic, they pose a infinite threat to human health care in terms of morbidity and mortality in both developed and developing countries [1, 2]. Subsequent to the serendipitous discovery of penicillin in the year 1928, when Alexander Fleming discovered the antibiotic penicillin from *Penicillium rubens*, antibiotics have been recognized as the only means of efficient to control the pathogenesis of microorganisms. In 1935, Gerhard Domagk developed the first synthetic antibacterial drug 'sulfonamide' with incredible clinical success in treating several microbial infections [3].

Along with the usage of new antibiotics as therapeutics, there is an emerging menace of drug-resistance among the pathogenic microorganisms worldwide. The indiscriminate use of antibiotics has led to an increase in resistance mechanisms among various pathogenic microorganisms and the present scenario necessitates the need for new alternatives to combat infections, especially those of bacterial and fungal origin [4]. This looming scenario is projected to reach catastrophic proportions by the year 2050, with antimicrobial resistance causing a distressing increase in morbidity and accounting for an estimated 10 million deaths yearly [5]. Moreover its devastating human toll, antimicrobial resistance also exacts a heavy levy on the global economy. In response to this critical issue, natural products have emerged as a promising device for combating antimicrobial resistance. Natural products offer a diverse array of chemical compounds and functional substances that have demonstrated their effectiveness against resistant pathogenic microorganisms. In quintessence, they represent an imperative alternative in the enduring battle against this global health threat [6]. Considering the urgency of the situation, the World Health Organization (WHO) has recognized the importance of Traditional, Complementary, and Integrative Medicine (TCIM) practices [7]. These approaches, rooted in ancestral experiences, provide valuable insights into disease prevention and recovery.

Even before the clinical use of antibiotics, Alexander Fleming's research group discovered a bacterial enzyme penicillinase that can inactivate penicillin [8, 9]. Mortality rates caused by multi-drug resistant bacterial and fungal infections have been reported to be quite high in both developed and developing countries. Scientists have warned that the world will return to a pre-antibiotic era plagued by life-threatening microbial infections on the basis of available antibiotic resistant gene database predicted from available genome sequences., Despite the progress made in the understanding of etiology, epidemiology, pathology and control the incidence of epidemics due to drug resistant microorganisms, the emergence of hitherto unknown disease causing microbes to pose critical problems in the treatment of public concerns [10]. Each nation must adopt a strategy to struggle antimicrobial resistance tailored to its conditions. This situation is graver in developing countries like India where easy availability, use of antibiotics in inappropriate high doses, and cost constraints to replace older antibiotics with new expensive antibiotics increase the probability of increased existence of antimicrobial-resistant strains. Hence there is an increased demand for the search of new lead molecules as antimicrobial agents. Rational drug design does not always result in potential antimicrobials. Most of the enzyme inhibitors that have been designed and synthesized in the past elicit only moderate antimicrobial activity probably owing to the complex issues associated with their uptake by the living cells and bioavailability [11].

Phytochemicals are ecologically derived secondary metabolites synthesized by the plants from the primary metabolites such as carbohydrates, lipids and amino acids to protect them against environmental challenges such as UV- irradiation, extreme cold, drought, microbial attack, wound, sugar and nutrient deficiency. They often contribute to the unique odor, taste and color in plants [12, 13]. Based on the chemical nature, the secondary metabolites are mainly classified into alkaloids, steroids, saponins, tannins, lectins, pectins, terpenoids, anthraquinones, flavonoids, glycosides and phenolic compounds. Fascinatingly,

these plant derived secondary metabolites are known to bring out significant pharmacological and beneficial effects to alleviate chronic communicable and non-communicable diseases [14, 15]. Recent advances in the field of medicinal chemistry lead to the discovery of isolating the active phytochemicals from various parts of the medicinal plants for treating human infectious diseases. Numerous structural analogs of phytochemicals have been successfully generated and widely used for their pharmacological actions [16]. However, the therapeutic efficacy of medicinal plant extracts/natural products is usually synergetic in nature [17]. Since earliest times, they have been known to exert significant antimicrobial properties against human pathogens. Around 40% of the natural products exist in the world have been subjected into pharmacological screening and a substantial number of new antibiotics derived from them have been introduced in the clinical practice [18].

Propolis is a complex mixture of resinous and balsamic substances of varied consistency, texture, and coloration collected by *Apis mellifera L*. bees or stingless bee species, from various parts of plants such as floral buds, shoots and resinous exudates in the vicinity of the apiary. The bees add salivary secretions, wax, and pollen, which readily accounts for the variation in its coloration, odor, texture and consistency [19]. Infact, Brazilian propolis has been classified into twelve classes with a diverse range of colors [20]. Propolis is a common ingredient used in apitherapy in various parts of the world. It is stated that propolis use dates back to ancient times, at least to 300BC, where it was used in folk medicine and other beneficial activities in many parts of the world [21]. It is one of the few natural products that maintained reputation for a long time in food and pharmaceutical industries [22, 23]. Propolis is a natural product that honeybees collect from several plants and mix it with beeswax and salivary enzymes [24, 25]. The term “propolis” derives from two terms of Greek origin, “pro” and “polis which literally mean “in front of or at the entrance of the city” [26]. Propolis is commonly used by the bees as building material and sealer by maintaining thermal homeostasis, waterproofing of the hive against moisture, reducing vibrations, averting the uncontrolled airflow into the nest, defend the colony against microbial infection and prevent putrefaction [27, 28].

Propolis is a lipophilic material melting at temperatures around 700 C [29]. It consists of granules of various sizes and with an enjoyable aromatic smell and different coloration, including red, brown, yellow and light green among others [30, 31]. Plant source, regional vegetation, seasons of harvesting, geography, type of bee flora, climate changes, physiochemical properties and antimicrobial activity are vital parameters that determine the quality of the propolis [32]. Ethanol is the best suitable solvent to extract the active principals from the propolis but is also used methanol, chloroform, ether and acetone [33 - 35]. According to a recent report, up to about 300 different components have been isolated, identified from different propolis samples [36 - 40].

Several studies performed on various samples evidenced that the main secondary metabolites are phenolic substances especially flavonoids, belonging to different sub-classes such as flavanones. flavones, flavonols and dihydroflavonols, which constitute more than 50% of the propolis weight [41, 42]. In addition, some non-phenolic compounds belonging to

different classes such as aliphatic acids, coumarins, aromatic hydrocarbons, terpenoids, steroids, esters, ketones, aldehydes, fattyacids, aminoacids, polysaccharides, hydrocarbons, hydroxybenzene and isoprenylated benzophenones have also been reported [43, 44]. Further, propolis is a rich source of minerals such as sodium, potassium, magnesium, calcium, vanadium, nickel, zinc, copper, iron, barium, strontium, cadmium, titanium, silver, molybdenum and cobalt [45-48].

Despite propolis popularity over time, it is not considered as a therapeutic agent in conventional medicinal system as the standardization of chemical composition and biological activities due to diversification of chemical composition are lacking and such consistency is indispensable for acceptable in the health system. Thus, characterization of different types of propolis according to its origin, chemical composition and biological activity is essential. Several reports are available in the literature evidencing the antioxidant, antiplatelet, anticancer, antimicrobial, anti-inflammatory and hepato-protective nature of propolis [49].

India is one of the largest countries in terms of environmental biodiversity, inhabiting large number of flora and fauna. The various climatic conditions and seasonal variations impart the qualitative and quantitative chemical composition and biological properties of Indian propolis. The study on Indian propolis has just started and only a few reports are available in the literature on the chemical composition and its beneficial as well as pharmacological properties. Earlier, we have reported the wound healing, anti-ulcerogenic properties of Indian propolis in experimental animal models [50]. More recently, we have reported the effect of seasonal variation in the antioxidant properties of Indian propolis [51]. The acute oral toxicity conducted in experimental rats revealed the non-toxic nature of the propolis. Fresh propolis samples have been widely used as an antimicrobial agent in traditional medicine. In the absence of systematic reports in the scientific literature regarding the antimicrobial activities of Indian propolis, the present study was aimed to screen the antibacterial and antifungal properties of propolis against clinically important bacteria and fungi.

MATERIALS AND METHODS

Collection of Propolis samples and preparation of extract

The raw propolis samples were collected from the apiary located in the protected area near Mudivaithaanendal, Vakaikulam, Thoothukudi District, Tamil Nadu, India using propolis traps and they were stored in the dark at -40C until their processing. The samples were collected during the month of June. The frozen samples were cut into small pieces, weighed and extracted with 10-fold volume of ethanol (95% v/v) under constant stirring overnight and centrifuged at 27,000 rpm for 15mins [52]. The supernatant was then concentrated until constant weight in a rotary evaporator under reduced pressure at 400C. All other chemicals, solvents, and reagents procured for conducting the present study were of analytical grade obtained from SRL, Mumbai.

Phytochemical screening

The ethanolic extract of propolis was subjected to phytochemical screening for the qualitative analysis of phytochemicals such as alkaloids, flavonoids, glycosides, saponins, pectins, tannins, phytosterols, triterpenoids, anthraquinones, and phenols [53, 54]. The experiments were conducted in triplicates to obtain concordant data.

Bacterial, fungal strains and growth medium

The bacterial and fungal strains used in the present study are standard laboratory strains procured from the stock cultures of the Division of Microbiology, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulattur, and maintained at 20°C on Muller Hinton Agar (MHA) (Himedia) and Potato Dextrose Agar (PDA) (Himedia) media for bacteria and fungus, respectively. The essential composition of the MHA media to maintain bacterial cultures includes beef extract, acid hydrolysate of casein, starch, and agar. The medium was prepared by adding 2 gm of beef extract, 17.50 gm of acid hydrolysate of casein, 1.50 gm of starch, and 17 gm of agar in one liter of distilled water. The final pH of the medium was adjusted to 7.3 ± 0.1 at 250C [55]. The composition of the PDA media used for fungal cultures comprises potato infusion, dextrose, and agar. Briefly, the medium was prepared by adding 200 gm of potato infusion, 20 gm of dextrose, and 20 gm of agar in one liter of distilled water, and the final pH was adjusted to 5.6 ± 0.2 [56].

The Gram-positive bacteria used in the present study include *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Streptococcus pyogenes,* and *Streptococcus pneumoniae,* and the Gram-negative bacteria include *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella dysenteriae,* and *Pseudomonas aeruginosa.* The fungal cultures chosen for the present study include Candida *albicans, Saccharomyces cerevisiae, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus, Penicillium chrysogenum,* and *Penicillium notatum.*

The bacterial cultures were maintained on slopes of MHA medium and sub-cultured every 15th day to prevent pleomorphic transformation. The bacterial cultures were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 106 to 108 CFU/ml. Likewise, the fungal strains were subcultured on slants of PDA at 280C for 7 days, and the colonies were suspended in 1 ml of sterile normal saline. The resulting mixture of conidia and hyphal fragments was vortexed, and the turbidity of each homogenous suspension was adjusted to match that of a 0.5 McFarland standard, as read at 530 nm. At this turbidity, the fungi density was maintained at 3 x 106 to 5 x 106 CFU ml^{-1}.

Determination of antibacterial and antifungal activity

Preparation of inoculums

The suspension for inoculation was prepared from the broth culture. Few colonies of similar morphology of the selected bacteria from twenty-four hours old culture were

transferred with the aid of a sterile inoculating loop to a Muller-Hinton broth and were incubated until adequate growth of turbidity equivalent to McFarland 0.5 turbidity standard (108 CFU/ml) was achieved. The turbidity was corrected by adding physiological saline. The isolates were sub-cultured on MH Agar and incubated at 350C for 7–14 days. The growth was scraped aseptically, crushed, and macerated thoroughly in sterile distilled water. Similarly, the fungal inoculums were prepared from 5- to 10-day-old cultures grown on PDA medium. The Petri dishes were flooded with 8 to 10 ml of distilled water, and the conidia were scraped using the sterile spatula. The spore density of each fungus was adjusted with spectrophotometer absorbance at 595 nm to obtain a final concentration of approximately 105 spores/ml. The fungal suspension was standardized spectrophotometrically to an absorbance of 0.600 at 450 nm.

Preparation of the McFarland standard

The preparation of McFarland Standard was carried out by mixing appropriate proportions of 1 ml of 36N sulfuric acid in 99 ml of water and 1% anhydrous barium chloride solution in 100 ml of water. The reaction between the two chemicals results in turbidity, which in turn is due to the formation of a fine precipitate of barium sulfate. The most commonly used 0.5 McFarland solutions as a standard for the Antibiotic Susceptibility Test (AST) were freshly prepared by mixing 0.5 ml $BaCl_2$ in 99.5 ml of 1% H_2SO_4 solution. The solution was shaken well so that the precipitate was distributed homogenously in the solution to obtain specific optical densities. A 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension of 1.5 x 108 colony-forming units (CFU/ml). The 0.5% McFarland turbid solution is used as a standard solution to which the cultures bacterial suspensions are compared and standardized. The approximate number of bacteria in a liquid suspension or broth culture was determined by comparing the turbidity of the cultured test suspension with that of the McFarland Standard [57].

The antibacterial activity of the ethanol extract of propolis samples were evaluated by the agar well diffusion method. The stock solution of the propolis extract (2.5 mg/ml) was prepared in sterile distilled water [58]. Dilutions of the stock solution containing 50, 100, 150, 200, and 250 mg were also prepared in sterile distilled water. The pure 24-hour-old bacterial cultures were aseptically transferred to a sterile saline solution into different 10 ml test tubes. They were matched with 0.5 McFarland standards.

The inoculums with respective bacteria to be tested were homogenously seeded onto the 90 mm Petri dishes containing 20 ml of cooled molten Muller Hinton agar medium using a sterile cotton swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. The inoculums were then spread evenly by using a spreader (sterile cotton swab). Thereafter, with the help of a 9mm sterile cork borer, the bores were made on the agar medium plates. Each concentration was marked at the back of the agar bores prior to filling. Using a sterile pipette, 100 µl of sterile distilled water was added to the control wells. Similarly, 100 µl of each dilution of the propolis extract was added into the wells. The plates were kept for 1 hr at room temperature to allow free diffusion

of the extract into the agar medium. Subsequently, all the plates were incubated at 37°C for 18-24 h. Following incubation, the plates were examined for signs of microbial growth. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. The diameters and the bore sizes were measured to the nearest mm. Chloramphenicol (30 µg/ml) was used as a positive control. Each experiment was carried out in triplicate.

Antifungal activity of the ethanol extract of propolis extract was evaluated by the disc diffusion method. The inoculums with respective fungi were homogenously seeded onto the 90 mm Petri dishes containing 20 ml of cooled molten SDA medium using a sterile pipette in such a way as to ensure thorough coverage of the plates and a uniform lawn of growth following incubation [59]. These inoculated plates were left to dry for at least 15 min. The propolis extract was dissolved in sterile distilled water to obtain the different concentrations of 0.175, 0.375, 0.75, 1.5, and 3 mg/disc. Amphotericin B at a concentration of 10 µg/disc was used as a positive control and was dissolved in dimethyl sulphoxide (DMSO). Sterile filter paper discs (6 mm in diameter) were impregnated with 10 µl of each different concentration of propolis extract. The discs were allowed to dry and then placed on the agar surface of each Petri dish. DMSO was used as a negative control. The zone of inhibition (in mm) was measured after 48-72 h at 28°C. The complete antifungal analysis was carried out under strict aseptic conditions. Each assay was repeated for a minimum of three times.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) assays

A serial 2-fold macro-broth dilution method was performed to determine the MICs and MBCs of propolis extract for the respective tested bacterial suspensions (concentration) as recommended by the Clinical and Laboratory Standards Institute (CLSI) [60]. The minimum inhibitory concentration (MIC) of propolis extract against the fungal strains was determined using the broth micro dilution method as described by the National Committee for Clinical Laboratory Standards for fungi (M27-A2). The stock solutions of propolis extract were diluted suitably as required from the stock solution. The ranges should be prepared one step higher than the final dilution range required (if a final dilution range of 0.5, 1, 2, 4, 8, and 16 mg/ml is required, then a range of 1, 2, 4, 8, 16, and 32 mg/ml should be prepared) to compensate for the addition of an equal volume of inoculums. Two rows of 12 capped test tubes were arranged in the test tube rack. In a sterile 30 ml universal screw-capped bottle, 8 ml of MH broth (bacteria) and 8 ml of PDA broth (fungi) containing the required concentration of propolis extract for the first tube in each row was prepared from the appropriate stock solution already made. The contents of the universal bottle were mixed using a sterile pipette and 2 ml were transferred to the first tube in each row. Using a fresh sterile pipette, 4 ml of broth was added to the remaining 4 ml in the universal bottle, mixed well, and 2 ml was transferred to the second tube in each row. Dilutions were continued in this way to as many as 10 tubes. Subsequently, 2 ml of broth free from extract was added to the last tube in each row. The density of the bacterial suspension was adjusted (108 CFU/ml) to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. The bacterial suspension was suitably diluted (106 CFU/ml) and added to the tubes

containing MH broth. Chloramphenicol (30 mg) was used as a positive control. After incubation at 37°C for 24 h, the turbidity of the tubes was assessed visually by comparison to uninoculated control.

Minimum fungicidal concentration (MFC)

To determine the MFC, Amphotericin B was included in the assays as a positive control (10 μg/disc) for fungi. After incubation at 28°C for 42-78 h, the turbidity of the contents in the tubes was assessed visually by comparison to uninoculated control. The MIC is expressed as the lowest concentration of the propolis extract where bacterial and fungal growth and fungal growth with no visible growth after incubation. All the assays were tested in triplicate.

RESULTS AND DISCUSSION

The continuous evolution of drug resistance to most of the currently available antimicrobial drugs has necessitated the search for novel and effective therapeutic agents, especially from natural resources. The development of antibiotic resistance is multifactorial, such as the unique nature of the microbes to antibiotics, indiscriminate use of various antimicrobial drugs, host characteristics, and environmental factors. Substances and extracts isolated from different natural resources, especially medicinal plants, have always been a rich arsenal for controlling the microbial infections and spoilage [61]. The combined effects of plant secondary metabolites are being studied in order to improve the antimicrobial activity and lessen the bad side effects of current antimicrobials.

The ethanolic extract of the propolis was filtered, dried, and weighed. The yield was around 8.5% w/w. Ethanolic extraction remains widely utilized [62 - 66]. The data obtained through qualitative analysis of phytochemicals such as alkaloids, flavonoids, glycosides, saponins, tannins, pectins, phytosterols, triterpenoids, phenols, and anthraquinones in the propolis extract. Nevertheless, the quality of propolis can be comprised by impurities, including beeswax residue, water content, ash and mechanical contaminants such as remnants of vegetation or bees, dyes and vegetable residues [67, 68].

In the present study, the antimicrobial activity of the ethanol extract of propolis was screened against five clinically important Gram-positive, five Gram-negative, and eight fungi. The efficacy was qualitatively and quantitatively evaluated by the diameter of the inhibition zones, minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) [69]. The data obtained were presented as tables 1, 2, 3, and 4 respectively. The zone of inhibition for both the bacteria and fungi was presented in mm, and the minimum bactericidal and fungicidal concentrations were assessed visually by comparison to uninoculated control. The findings were compared with the growth inhibition results obtained for the standards (Chloramphenicol for the bacteria and Amphotericin B for fungi).

The agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. When compared to the disc diffusion method, the agar well diffusion method is considered to be superior because of the fact that the antimicrobial agent diffuses freely in the solid nutrient medium and inhibits the growth of the microbial strains [70]. The disc diffusion test, or agar diffusion test, or Kirby–Bauer test, is a test of the antibiotic sensitivity of fungi. Briefly, it uses the antibiotic discs to evaluate the extent to which fungi are affected by selected antibiotics. In this test, wafers containing the antifungal agents are placed on an agar plate where the fungus has been streaked. The area around the wafer where the fungus has not grown enough to be visible is called a zone of inhibition [71].

Chloramphenicol (CAM), a well-known antibacterial drug, was originally isolated in 1947 from *Streptomyces venezuela* and was introduced in clinical practice in 1949 [72]. CAM is effective parenterally as well as orally and has excellent cell penetration potential. It consists of a p-nitrobenzene moiety, a 2-amino-1, 3-propanediol moiety, and a dichloroacetyl tail. CAM is active against a broad spectrum of bacteria, usually behaving as a bacteriostatic drug, although it exhibits bactericidal activity against the most common causes of meningitis, Haemophilus influenza, Streptococcus pneumonia, and Neisseria meningitides [73]. CAM selectively inhibits protein synthesis by binding to the peptidyl transferase (PTase) center of the bacterial ribosome and abrogating essential ribosomal functions like peptide-bond formation [74] termination of translation [75] and translational accuracy [76].

Amphotericin B (AMB) was used as a reliable standard drug to compare the antifungal activity of the propolis extract. AMB is considered a reference drug in evaluating the antifungal activity of unknown drugs developed for the treatment of serious invasive mycoses. It is a polyene antifungal agent, and its mode of action is based on the formation of a complex with the ergosterol in the fungal cell membrane, causing destabilization and subsequent release of a vital cell component [77, 78]. Liposome-encapsulated AMB's lower affinity for mammalian cells and its enhanced distribution volume readily account for its decreased toxicity and the broad range of antifungal efficacy [79, 80].

Table 1 shows the antibacterial activity of propolis extract against the Gram-positive and Gram-negative bacteria. From the results, it is evident that the ethanolic extract of propolis showed an inhibitory zone in a dose-dependent manner. However, there was no significant difference between the levels of the zone of inhibition at the concentrations of 200 µg and 250 µg. The sensitivity of the propolis extract was found to be in the order of *Bacillus subtilis* > *Streptococcus pyogenes* > *Staphylococcus epidermidis* > *Streptococcus pneumoniae* and *Staphylococcus aureus* against the Gram-positive bacteria and *Klebsiella pneumoniae* > *Shigella dysenteriae* > *Salmonella typhi* > *Escherichia coli* and *Pseudomonas aeruginosa* for the Gram-negative bacteria. Antimicrobials are classified based on a variety of methods, such as spectrum of activity, effect on microbes, and mode of action. Based on the nature of their effect on bacteria, antibiotics are classified as bactericidal (lysis) and bacteriostatic (inhibiting bacterial growth and replication). However, the effect of bactericidal agents is faster as compared to bacteriostatic agents. Conversely, some antibiotics may behave as both bacteriostatic and bactericidal based on dosage and duration.

The mode of action of antimicrobials differs on the basis of the nature of their structure and degree of affinity to target sites within the bacterial cells, which results in the inhibition of cell wall synthesis, cell membrane functions, and protein synthesis. Christian Gram in 1884 developed a staining procedure that allowed him to classify nearly all bacteria into two broad groups, and this eponymous stain is still in widespread use. One group of bacteria retains Christian's stain, Gram-positive, and the other does not, Gram-negative. Gram-positive bacteria show blue or purple after Gram staining due to the presence of a thick peptidoglycan cell wall (20-80 nm) along with teichoic acid. Gram-negative bacteria show pink or red staining and have a thin peptidoglycan cell wall with no teichoic acid. Thus, the basis for the Gram stain lies in fundamental structural differences in the cell envelope of these two groups of bacteria. Escherichia coli are gram-negative and are used as the model organism in most bacterial studies. Staphylococcus and Streptococci are examples of gram-positive bacteria. The antibacterial activity of propolis differs between Gram-positive and Gram –negative bacteria, primarily due to distinctions in the structure and arrangement of the cell wall although the action is more pronounced against Gram-positive bacteria [81]. Thus, the observed difference in the sensitivity of the propolis extract among the different bacteria may be due to morphological differences between them. Further, the Gram-positive bacteria were known to be more vulnerable since they possess only an outer peptidoglycan layer, which is not an effective permeability barrier and may facilitate the infiltration of hydrophobic compounds [82]. Further, the observed significant antibacterial activity may be attributed to multiple targets, with various constituents such as phenolic compounds, diterpenes and flavonoids present in the propolis extract which acts synergistically [83].

Table 1: Antibacterial activity of propolis extract- Zone of inhibition in diameter (mm)

S. No.	**Bacterial species**	**Control**	**50 μg**	**100 μg**	**150 μg**	**200 μg**	**250 μg**	**Chloramphenicol (30 μg)**
Gram Positive								
1.	*Staphylococcus aureus*	-	2.8	5.2	9.5	14.5	19.0	26.0
2	*Bacillus subtilis*	-	4.5	9.0	13.0	25.0	29.0	29.0
3	*Staphylococcus epidermidis*	-	4.0	8.5	13.5	18.5	23.0	27.0
4	*Streptococcus pyogenes*	-	4.5	8.5	15.5	21.0	22.5	26.0
5	*Streptococcus pneumoniae*	-	2.5	4.0	9.5	14.5	19.0	24.0
Gram Negative								
6	*Escherichia coli*	-	2.0	3.0	7.0	15.5	18.0	24.0
7	*Klebsiella pneumoniae*	-	3.5	6.0	10.0	12.0	17.0	28.0
8	*Salmonella typhi*	-	2.0	3.5	7.5	10.0	16.0	26.0
9	*Shigella*	-	4.5	7.5	14.5	12.0	17.0	24.0

	dysenteriae							
10	*Pseudomonas aeruginosa*	-	3.5	5.5	9.5	17.5	18.0	21.0

Fungi are a diversified group of microorganisms that are present in the environment, being a part of the normal flora of humans and animals, and have the ability to cause injury ranging from mild superficial infections like “jock itch” to severe life-threatening invasive infections such as cryptococcal meningitis. The diseases caused by the fungus are termed ‘Mycotoxicoses.’ The term ‘antifungal’ encompasses all chemical compounds, pharmacological agents, and natural products used to treat mycoses. Clinically, fungal infections are categorized according to the site and extent of the infection, route of acquisition and the virulence of the causative organism. These classifications are essential when determining the most effective treatment regimen for a particular mycosis. Mycoses are classified as local (superficial, cutaneous, subcutaneous) or systemic (deep bloodborne). The acquisition of the fungal infection is either an exogenous (airborne/inhalation, cutaneous exposure, percutaneous inoculation) or an endogenous process (normal flora or reactivated infection).

The virulence of the organism is classified as either a primary infection (disease arising in a healthy host) or opportunistic infection (disease arising in human hosts that have a compromised immune system or other diseases). There are three main classes of systemic antifungals, namely the polyene macrolides, the azoles, and the allylamines. Amphotericin B deoxycholate, a polyene antibiotic, was the first antifungal agent introduced in 1958 to treat systemic mycoses. While this drug is an effective agent, the demand for other efficacious topical, oral, and intravenous agents was apparent. Griseofulvin was introduced in 1959, representing the second class of antifungals. Flucytosine, the antimetabolite drug, entered the market in the year 1971. While the antimycotic pharmacology has advanced significantly, particularly in the last three decades, common invasive fungal infections still carry a high mortality rate: Candida albicans (approximately 20-40% mortality), Aspergillus fumigatus (approximately 50-90%), and Cryptococcus neoformans (approximately 20-70%) [84-86].

The antifungal activities of the propolis extract against the selected pathogenic fungi are presented as Table 2. The data obtained evidenced that the fruits extract showed antifungal activity similar to that of antibacterial activity. The highest antifungal activity (diameter of the zone of inhibition 24 mm) was demonstrated against Candida albicans, while the lowest activity was observed against Saccharomyces cerevisiae. The results of the in vitro antifungal assay also revealed that the growth of fungal strains was severely affected by the propolis extract by forming clear inhibition zones. The antibacterial and the antifungal activity of the propolis extract were comparable with Chloramphenicol and Amphotericin B, respectively. The antifungal activity of propolis primarily arises from its phenolic compounds [87,88]. These compounds thwart fungal growth by interacting with the cell wall and plasma membrane leading to heightened permeability and produces extravasation of sodium, potassium and hydrogen ions, causing the fungus to die [89]. At the mitochondrial level, they induce alterations in the electron transport chain, ultimately induce apoptosis and prolonged

exposure exacerbates this effect, resulting in secondary necrosis [90]. In addition, propolis stimulates reactive oxygen species (ROS) generation and interferes with the calcium signaling pathways [91].

Table 2: Antifungal activity of propolis extract against selected fungal species determined by disc diffusion assay.

Sl. No.	Strains	Control	0.175 mg/disc	0.375 mg/disc	0.75 mg/disc	1.5 mg/disc	3 mg/disc	Amphotericin B
1	*Candida albicans*	-	4.5	8.5	14.5	17.5	21.0	26.0
2	*Saccharomyces cerevisiae*	-	-	6.5	9.0	15.5	18.0	21.0
3	*Aspergillus fumigatus*	-	9.0	13.5	16.0	18.5	22.0	24.0
4	*Aspergillus flavus*	-	7.5	13.5	16.0	21.0	23.0	25.0
5	*Aspergillus niger*	-	7.5	9.0	13.0	19.5	22.0	24.0
6	*Aspergillus ochraceus*		7.5	10.0	13.5	18.5	21.5	26.0
7	*Penicillium chrysogenum*	-	6.0	9.5	13.0	17.0	21.0	23.0
8	*Penicillium notatum*	-	11.0	14.5	17.5	21.5	23.5	25.0

The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and the minimum fungicidal concentration (MFC) of propolis extract as well as the standard antibiotics are shown in **Tables 3 and 4**. The MIC, MBC, and MFC may be defined as the minimum concentration of the antimicrobial agent, which exhibits the maximum zone of inhibition and allows no visible growth, respectively. The MIC values of fruits extract against both the Gram-positive and the Gram-negative bacterial strains vary from 1 to 5 mg, and the efficacy was comparable with the standard drug. However, the lowest MIC values were shown by *Bacillus subtilis* in Gram-positive bacteria and by *Klebsiella pneumoniae* in Gram-negative bacteria. The highest MIC values were shown by *Staphylococcus aureus* in Gram-positive bacteria and by *Salmonella typhi* in Gram-negative bacteria. Likewise, the MBC values also represent the significant antibacterial activity of the propolis extract **(Table 28).**

Table 3: MICs and MBCs of propolis extract on Gram positive and Gram negative bacteria.

Bacterial species	Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	Propolis extract (mg/ml)	Chloramphenicol (µg/ml)	Propolis extract (mg/ml)	Chloramphenicol (µg/ml)
Gram positive				
Staphylococcus aureus	6	5	4	3
Bacillus subtilis	2	3	4	5
Staphylococcus epidermidis	3	4	5	5
Streptococcus pyogenes	5	5	3	3
Streptococcus pneumoniae	3	3	2	2
Gram negative				
Escherichia coli	4	3	6	8
Klebsiella pneumoniae	2	3	2	3
Salmonella typhi	3	3	6	7
Shigella dysenteriae	3	3	4	5
Pseudomonas aeruginosa	3	4	4	6

The fungal strains used in the present study to evaluate the antifungal activity of the propolis were selected on the basis of their clinical importance. Among the fungi, the lowest MIC values were shown by *Candida albicans*, and the highest MIC value was elicited by *Saccharomyces cerevisiae* (Table 29). It is pertinent to note that the Candida infections have been associated with the highest rates of morbidity as well as associated mortality of more than 38% [92,93].

Table 4: Antifungal activity of propolis extract against fungal species determined by MIC and MFC.

Fungal species	MIC		MFC	
	Propolis extract (mg ml^{-1})	Amphotericin B (µg ml^{-1})	Propolis extract (mg ml^{-1})	Amphotericin B (µg ml^{-1})
Candida albicans	2.5	2.0	2.0	3

Saccharomyces cerevisiae	6.0	3.0	7.5	5
Aspergillus fumigatus	4.5	1.8	5.0	6
Aspergillus flavus	3.5	3.0	4.5	5
Aspergillus niger	5.5	2.5	5.5	4
Aspergillus ochraceus	4.5	2.0	4.5	2
Penicillium chrysogenum	3.0	3.5	4.0	2
Penicillium notatum	4.0	6.0	4.5	4

Though in vitro antimicrobial assays represent an alternative method, as they have been used successfully to identify promising treatment regimens for both bacterial and fungal infections [94-96], they have several limitations, such as the efficacy of the simulated antimicrobial regimens being tested in an artificial medium that does not necessarily reflect in vivo conditions or account for the potential contribution of the host immune response. Likewise, the applicability of test variables selected for the testing of antimicrobial agents to actual in vivo infection is not fully understood. Further, the concentrations of individual phytochemicals may vary in different plants, which results in unique medicinal properties for a specific plant [97]. The mode of preparation of sample extracts has also been linked to their antimicrobial properties [98]. Thus, it is necessary to isolate the active ingredients present in the propolis and evaluate their antimicrobial properties by various methods to develop successful antibiotics.

CONCLUSION

The assorted chemical composition of propolis influences by various sources, geographic regions, method of extraction underlines the significance of understanding the variations to formulate its beneficial and therapeutic applications. Indian propolis possess significant anti bacterial properties, especially against Gram-positive bacteria and has also shoe\wn significant antifungal properties. The observed antimicrobial efficacy of the Indian Propolis is mainly due to the presence of biologically important secondary metabolites, especially phenolic compounds, which functions synergistically via mechanisms affecting cell membranes and interfere with cellular functions. The results of the present study provide a new dimension for addressing the proplem of drug resistance to antimicrobial drugs and provide a scientific basis that the propolis extract might open new promising opportunities for the development of more efficient, non-toxic, and cost-effective natural antimicrobial agents for the control of various pathogenic microorganisms in the food and pharmaceutical industries and new clinically effective antimicrobial agents. Indian Propolis may be considered a potential source for the extraction, isolation, and identification of novel antimicrobial agents.

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