



Characterization, Antimicrobial and Anti-motility Activity of North Indian Propolis

Meenu Khurana^a, Anjali Sharma^b, Rupinderjeet Kaur^b, Tamanna Kaundal^a and Navneet Batra^{a*}

^aDepartment of Biotechnology, GGSD College, Sec 32, Chandigarh

^bDepartment of Biotechnology, DAV College, Sec 10, Chandigarh

Corresponding author (batranavneet@gmail.com)

ABSTRACT

The emergence of antibiotic resistance in present era has forced the scientific community to find the new alternatives in natural products. Numerous natural products are known to have the antimicrobial activity, one of them is Propolis. Propolis is bee glue made by honey bee as a sealant in the hives which protects the hives from parasites as well as prevents bacterial and fungal growth. About 300 constituents are known to be a part of Propolis mainly contains the phenols, flavonoids, terpenes, cumaric acid and esters. The bioactivity of propolis greatly varies with the geographical region, botanical sources and the bee species. The current study is aimed at characterizing the North Indian Propolis for its phenol, flavonoids content and antioxidant activity. The minimum inhibitory concentration was calculated against the planktonic microbes. The anti motility activity was studied for the microbes at sub lethal concentrations. It was observed that Propolis has antimicrobial and anti motility activity against all the microbes used in the present study.

Key words: Propolis, phenol, flavonoids, antioxidant, antimicrobial, anti motility

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INTRODUCTION

The discovery of antibiotics proved a boon to the human health and increased the longevity, but with increased use of antibiotics there is emergence of antimicrobial resistance. Antimicrobial resistance hampers the cost of infection control, surgeries, and raises cost of treatment. For example the resistance to penicillin developed in *Staphylococcus aureus* decreased the efficacy of drug against the staphylococcal infections in hospitalized patients where the resistant strains are frequently found. The mechanism behind can be single genetic mutation lead to resistance without altering the viability of bacterial strain. The bacteria also acquire genetic material from outside that also contributes to antimicrobial resistance. Many resistant genes are found in plasmid and transposons that are transferred to bacteria by conjugation and transposition that is referred to lateral gene transfer[1]. Also the resistant genes are present in natural population but the phenotypic effect is not visible until the use of drugs provides the selection pressure and cause differential reproduction favoring the growth of resistant microorganism.

The problem of resistance to drugs has limited the use of antibiotics, as we cannot administer the high levels of drug as it becomes toxic. In addition antibiotics kill the large portion of normal microbial community found in the body that is beneficial for the organism. The limitations of antibiotics have forced to find the new ventures in natural product that are non toxic. There are numerous natural products already known to have the antimicrobial effect. Usnic acid, a metabolite from lichens, has antimicrobial activity, against *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*, against a variety of planktonic gram-positive bacteria [2]. Berberine is natural isoquinoline alkaloid from various Chinese herbs, plant extracts from those from *Abies canadensis*, *Albizia julibrissin*, *Chelidonium majus*, *Ginkgo biloba*, *Juniperus virginiana*, *Pinus virginiana*, *Rosmarinus officinalis*, *Sassafras albidum*, *Tanacetum vulgare* and *Thuja plicata* inhibit the growth of oral streptococci. Garlic, *Allium sativum*, *Harungana madagascariensis* native African plant, resin exuded by the *Pistacia lentiscus*, ethanol extracts of *Piper cubeba* (Piperaceae) have also been known to have antimicrobial properties[3]. One of the natural animal product found to very efficient is Propolis .

Propolis is bee glue, red to dark brown sticky substance made by honey bee for sealing the gaps in the hives to prevent the entry of parasites as well as inhibit the fungal and bacterial growth[4]. It is synthesized by combining saliva, bee wax and exudates from tree buds, flower sap and other botanical sources. Normally the color is brown but green, red black and white color has been known in earlier

studies[5].It is a resinous mixture of about 300 different compounds, including phenols, flavonoids, lignans, terpenene, caffeic, ferulic and cumaric acids and esters etc [6]. The composition varies with geographical origin, botanical source and bee species[7]. Propolis is known for its diverse properties such as anti-microbial, antioxidant, antiviral, anticancer, antidiabetic, immunostimulatory[8].

Due to increase in cases of antibiotic resistance especially in case of hospital acquired infections. There is a great need of natural antimicrobial substances that has less toxicity and resistance. There are number of natural product known to have antimicrobial properties. Propolis is known to be one of them having known antimicrobial potential. Microcalorimetric and electron microscopic studies on the antibacterial mode of action of propolis against Streptococcus agalactiae have been published [9]. Propolis has been reported to suppress bacterial growth by blocking cell division, resulting in pseudo-multicellular streptococci development. In addition, cytoplasmic membrane, and cell wall were disorganized by propolis, which induced partial bacteriolysis, and inhibit protein synthesis.It was evident that the mechanism of inhibition of propolis on bacterial cells is complex and a simple analogy cannot be made to the mode of action. Propolis has shown to synergize antimicrobial action against Staphylococcus aureus with antibiotics that interfere with the synthesis of bacterial proteins[10].As the composition of propolis greatly varies with geographical area and botanical sources the present study was undertaken to characterize the antimicrobial potential of North Indian Propolis.

MATERIAL AND METHODS

Propolis extraction

Propolis was obtained from apiary near Chandigarh. The propolis used is of *Apis mellifera* which mainly feed on poplar vegetation in rainy season. It was finely grinded using mortar and pestle. The 30 gm of propolis was taken in 100 ml of 96% ethanol followed by continuous stirring for 7 days in dark bottle and filtered[11]. In order to remove wax from propolis extract it was kept at -20⁰C for 72 hours and then was filtered using 2µm filter[12]. The filtrate was dried and resinous propolis was stored at -20 ° C.The absorption spectrum for Propolis was obtained[13].

Estimation of total phenol content

Determination of phenols was performed using Folin- Ciocalteu reagent and calibration curve with correlation coefficient and standard error of estimate realized using Gallic acid as internal standard. The propolis extract was added to 5ml Folin- Ciocalteu reagent and Sodium carbonate (4ml 0.7M) and mixture was kept for 1 hour at room temperature. The absorption was measured at using UV-visible spectrophotometer 765 nm against the blank[14]. The standard curve was prepared using the 50-250 µg/ml solution of Gallic Acid. Total phenolic content in Propolis extract was expressed as Gallic Acid equivalents

$$T = (C \times V) / M$$

T = Total content of phenolic compounds (mg of GAE /g sample)

C= concentration of established Gallic acid from calibration curve (mg/ml)

V= Volume of extract

M= weight of propolis (gm)

Estimation of Flavonoid content

Determination of flavones /flavonols was performed using AlCl₃, and calibration curve with Correlation coefficient and standard error of estimate realized using Quercetin as internal standard. Propolis extract was added to 3ml of methanol, 0.2ml of 10% Aluminum chloride, 2ml of potassium acetate (1M) and 5.6 ml of distilled water. The solution was kept at room temperature for half an hour.The absorbance was measured at 415nm using UV visible spectrophotometer against the blank[15].The standard calibration curve was made using 25-200µg/ml of Quercetin. The flavonoid content was expressed in Quercetin Equivalent.

$$T = (C \times V) / M$$

T = Total content of flavnoid compounds (mg of QE /g sample)

C= concentration of Quercetin established from calibration curve (mg/ml)

V= Volume of extract

M= weight of propolis (gm)

Radical scavenging Activity using DPPH

Free radical scavenging activity of ethanol extract of propolis was measured by 1,1-diphenyl-2-picrylhydrazl (DPPH)[16].3Mm solution of DPPH was prepared.1ml of .3mM DPPH was added to 2.5ml of ethanolic propolis extract at different concentration.(50-1000µg/ml). The mixture was mixed and was kept at room temperature for half an hour then absorbance was observed at 518nm using spectrophotometer.

DPPH Scavenging effect or Percent inhibition = $\frac{A_0 - A_1}{A_0} \times 100$

A_0

A_0 = Absorbance of control

A_1 = Absorbance of test sample

Microorganisms: Total of six microorganisms used were obtained from MTCC. The Gram positive organism were *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC2470), Gram negative were *Escherichia coli* (MTCC 47), *Enterobacter cloacae* (MTCC 9145), *Pseudomonas aeruginosa* (MTCC 424) and fungus used was *Candida albicans* (MTCC 227).

Antimicrobial Activity

Antimicrobial activity was determined by agar diffusion and microdilution method [17]. For agar diffusion the test organism was inoculated in a media and incubated for 18 hours. The incubated culture was diluted with the fresh autoclaved media to compare the test suspension with the 0.5 Mc Farland standard in the presence of good light. The 50 μ l of the diluted media was spread on the agar plate. The well was made in the agar plate and the different concentration of propolis was loaded in the well along with the positive (antibiotic) and the negative control (ethanol).

The microdilution method was also performed to determine the MIC. The 100 μ l of NB (YEPD for *C. albicans*) media and 50 μ l of different concentration of propolis was added in well [18]. 10 μ l of .01% resazurin solution was added as indicator. 10 μ l of bacterial suspension (5×10^6 cfu/mL) was added in each well. Each plate was wrapped with paper loosely so that bacteria did not become dehydrated. Each plate had a set of positive and negative controls. The three plates were prepared, and placed in an incubator set at appropriate temperature. The colour change was observed visually. Any changes of color from purple to pink or colourless were recorded as positive. The lowest concentration of Propolis at which colour change was observed was considered as the MIC value. The average of three values was calculated and that was the MIC for microorganism.

Anti-motility Activity

Anti-motility assay was studied for all organisms in 0.34% agar consisting sub lethal concentration of propolis using Nutrient agar for *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Enterobacter cloacae*, LB agar for *Pseudomonas aeruginosa* and YEPD agar for *Candida albicans* [19]. 25 ml of the combined media was added to 9 cm Petri dishes. The plates were dried for 30 mins in a laminar hood. 10 μ l of bacterial culture was spotted onto the center of a plate. The control plates contained only ethanol without propolis and were kept at appropriate temperature for incubation for 24 hours.

RESULTS AND DISCUSSION

Propolis have number of biological active compounds as its constituents, the advantage of propolis is that it is non toxic over other antimicrobial agents. The compounds in propolis are already known to inhibit the glucosyl transferase enzyme in bacteria *Streptococcus mutans* [20]. The extraction characterization and antimicrobial activity of propolis extracted from various places like Portugal, Indonesia, Brazil, Toronto, Turkey, Iran, Philippines, Serbia, Romania has already been studied. In India the antimicrobial activity of propolis extracted from a place in Tamil Naidu against *Candida* and *Staphylococcus* has been already studied [21]. The characterization and antimicrobial and antibiofilm activity of Northern India Propolis has not been reported so far. Our aim was to characterize the propolis obtained from a region in North India for its phenol, flavonoids content and antioxidant activity and to find its antimicrobial activity on both Gram positive and Gram negative bacteria and also on *Candida albicans* which are all involved in hospital acquired infections.

Propolis extracted showed a characteristic absorption at 270-300 nm as shown in Figure 1 congruent with already studies done. The maxima absorbance is at 300 nm showing that the Propolis has been extracted. This absorbance is mainly due to the presence of phenolic acids and flavonoids that are important constituents of Propolis.

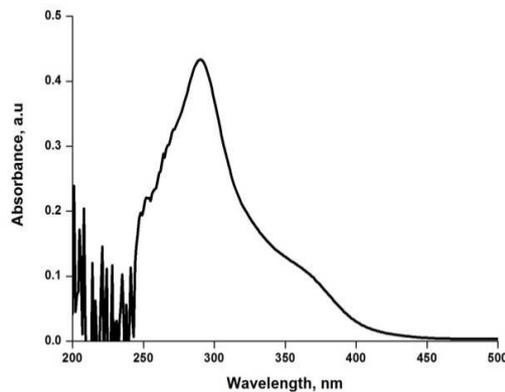


Figure 1. Absorption spectrum of extracted propolis

Phenol content was estimated in the Propolis employing the spectrophotometric assay using gallic acid as depicted in Fig 2. The phenol content in the Propolis extracted was estimated to be 144.6 mg GAE /g ($R^2=0.909$). The amount of polyphenols in Propolis which is already reported ranges from 30-200mgGAE/mg although as high as 500mg GAE/g has also been observed. The phenol content in the extract was estimated to be 144.6 mg GAE /g using the standard curve of gallic acid ($R^2=0.909$) (Figure 2).

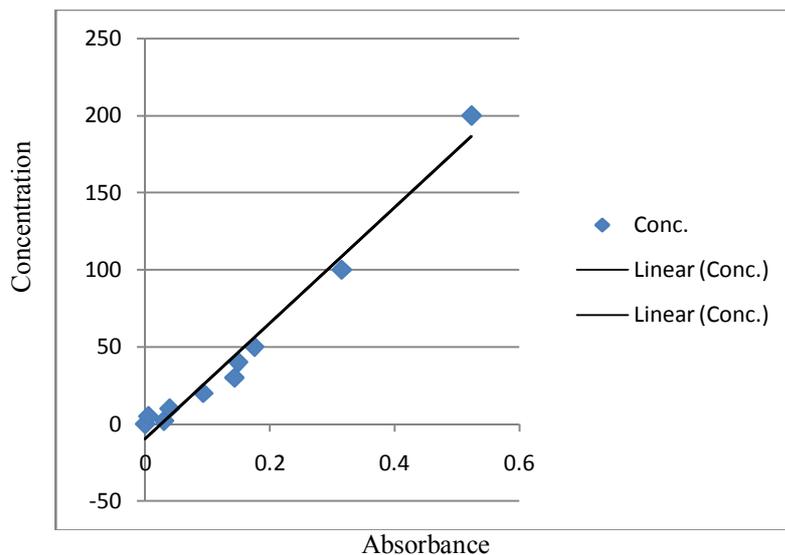


Figure 2. Estimation of Phenol content of Propolis

$R^2= 0.909$ was obtained by, by plotting the absorbance of different concentrations of Gallic acid.

$$y= 0.003 x +0.048$$

Using the standard curve generated by Quercetin ($R^2=0.945$) the total flavonoid content was estimated to be 69.6mg QAE/g (Figure 3). Using the standard curve generated by quercetin ($R^2=0.945$) the total flavonoids content was estimated to be 69.6mg QAE/g as depicted in Fig 3. Averagely the flavonoids content estimated in earlier studies is 30-70 QAE/g. The maximum content of flavonoids as 379mg QAE has already been reported [23].

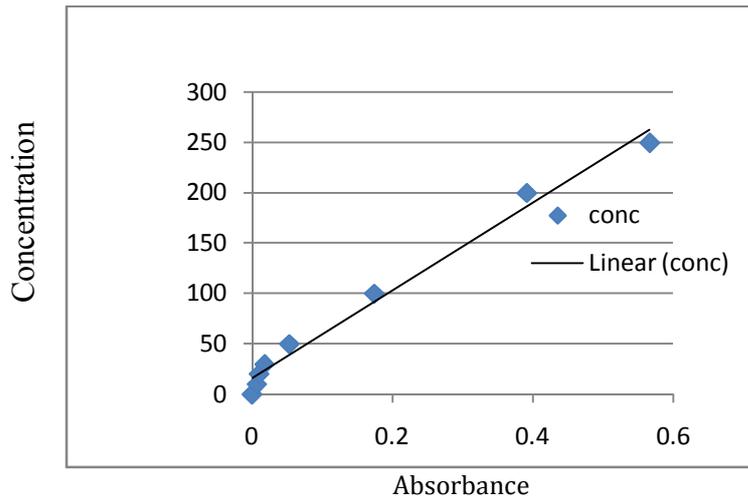


Figure 3. Estimation of flavonoids content of Propolis

$R^2 = 0.945$ was obtained by plotting the absorbance different concentrations of Quercetin
 $y = 0.002x + 0.041$

The flavonoid content in the extract was estimated to be 69.6 mg QAE /g

Using the standard curve generated by quercetin ($R^2 = 0.945$) the total flavonoids content was estimated to be 69.6 mg QAE/g. The radical scavenging effect for this North Indian Propolis was found to be 68.8% for the ethanolic extract. Studies from various regions of the world have reported the radical scavenging activity as 70.69 % for water extract and 82.44% for ethanolic extract of Propolis [24]. A radical scavenging activity of 92.4% has been cited in literature for red Propolis [25]. The difference in the composition of Propolis depends on the bee species, climate conditions, temperature, geographical area, vegetation, season of collection greatly contribute to biological activity of Propolis.

The antimicrobial assay was performed using the well diffusion (Figure 4) and micro dilution method using resazurin (Figure 5). The MIC found by both methods was found to be same as depicted in Table 1.

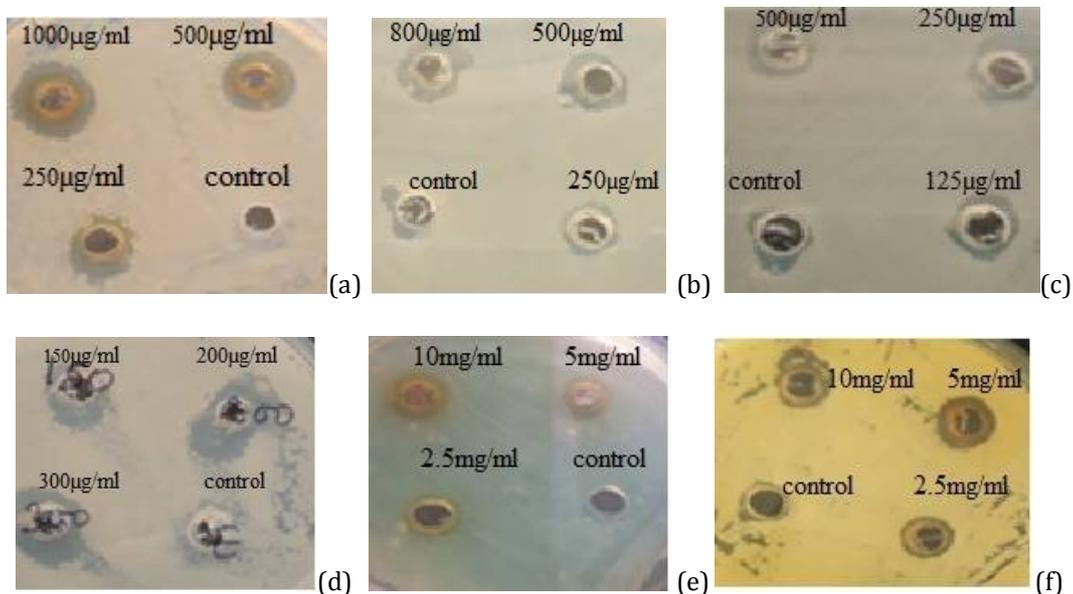


Figure 4. Antimicrobial activity of propolis (a) *Staphylococcus aureus* (b) *Micrococcus lutens* (c) *Escherichia coli* (d) *Enterobacter cloacae* (e) *Pseudomonas aeruginosa* (f) *Candida albicans*

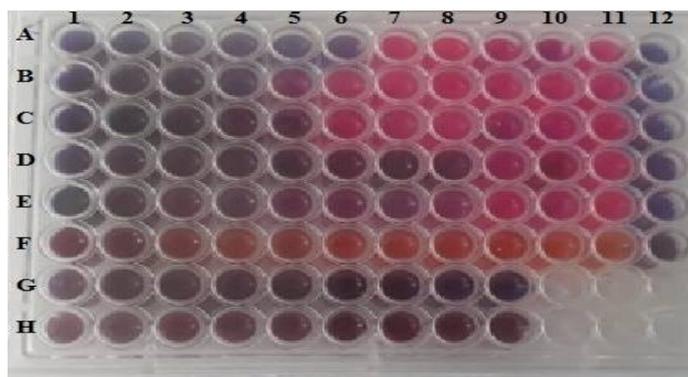


Figure 5 Determination of MIC by microdilution method.

Table 1 MIC of Propolis on different microorganisms obtained by disc diffusion and microdilution method

S. No.	Name of the microorganism	MIC
1	<i>Staphylococcus aureus</i>	250 µg/ml
2	<i>Micrococcus luteus</i>	250 µg/ml
3	<i>Escherichia coli</i>	125 µg/ml
4	<i>Enterobacter cloacae</i>	60 µg/ml
5	<i>Pseudomonas aeruginosa</i>	2.5mg/ml
6	<i>Candida albicans</i>	2.5mg/ml

A- *S.aureus* , B- *M.luteus* ,C- *E.coli* ,D- *E.cloacae* ,E-*P.aeruginosa* ,F-*C.albicans* ,G-NB+ propolis, H- YEPD+propolis ,1-9 different conc. Of propolis (50ug-10mg/ml), 10- culture+ ethanol,11- culture ,12- positive control.

The motility of all organisms was found to be affected as shown in the reduction in the zone of Motility (Figure 6 a-f).

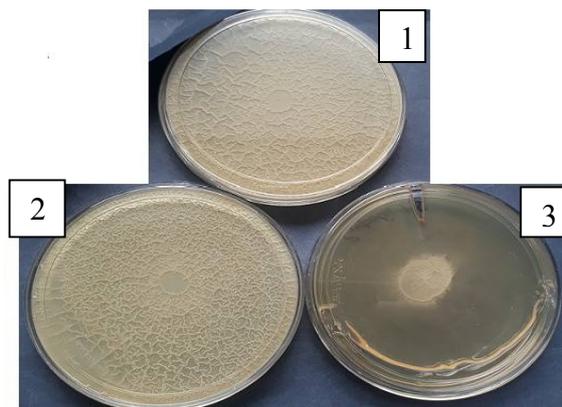


Figure6(a) showing the inhibition of motility in *Staphylococcus aureus*
 Plate1 *Staphylococcus aureus* grown on NA
 Plate 2 *Staphylococcus aureus* grown in presence of ethanol
 Plate 3 *Staphylococcus aureus* grown in presence of propolis below the MIC



Figure6 (b) showing the inhibition of Motility *Micrococcus luteus*
 Plate 1 *Micrococcus luteus* grown on NA
 Plate 2 *Micrococcus luteus* grown in presence of ethanol
 Plate 3 *Micrococcus luteus* grown in presence of propolis below the MIC

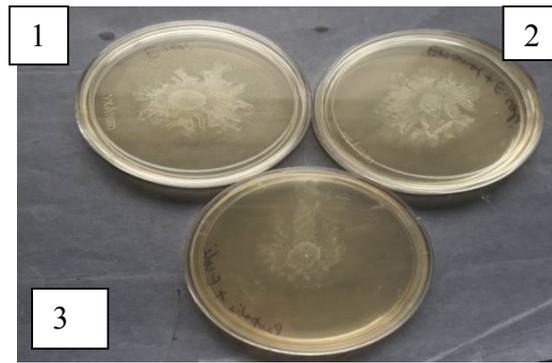


Figure6 (c) showing the inhibition of Motility in *E.coli*
Plate1 *E.coli* grown on NA plate
Plate 2 *E. coli* grown in presence of ethanol
Plate 3 *E. coli* grown in presence of propolis below the MIC

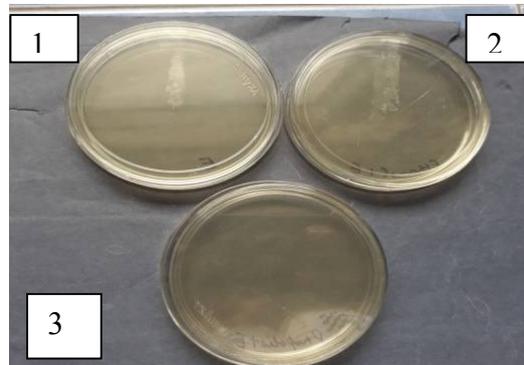


Figure6(d) showing the inhibition of Motility in *Enterobacter cloacae*
Plate1 *Enterobacter cloacae* grown on NA
Plate 2 *Enterobacter cloacae* grown in presence of ethanol
Plate 3 *Enterobacter cloacae* grown in presence of propolis below the MIC

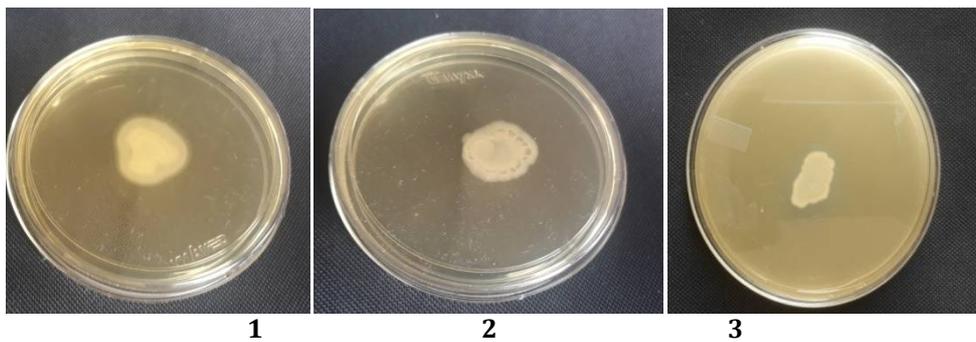


Figure 6(e) showing the inhibition of Motility in *Pseudomonas aeruginosa*
Plate1 *Pseudomonas aeruginosa* grown,
Plate 2 *Pseudomonas aeruginosa* in presence of ethanol
Plate 3 *Pseudomonas aeruginosa* grown in presence of propolis below the MIC

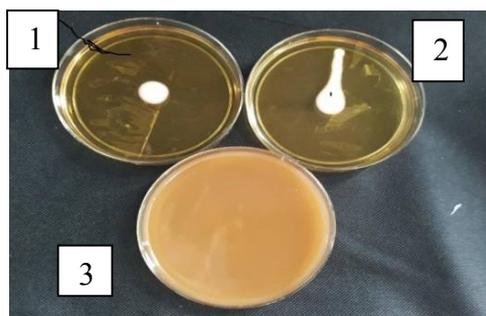


Figure 6(f) showing the inhibition of Motility in *Candida albicans*.
 Plate1 *Candida albicans* grown on YEPD
 Plate 2 *Candida albicans* in presence of ethanol
 Plate 3 *Candida albicans* grown in presence of propolis below the MIC

The comparative MIC of Indian Propolis from Tamil Naidu region shows maximum effectiveness at concentration of 1mg/ml against Gram +ve and Gram -ve bacteria [26]. Antimicrobial activity of Propolis is mainly due to the phenols, flavonoids. The study showed moderate efficacy of Propolis for Gram positive and Gram negative bacteria with the MIC ranges from 64- 250 μ g/ml except for *Pseudomonas aeruginosa* and *C.albicans* which displayed high resistance towards Propolis with MIC of 2.5mg/ml as reported in earlier studies but MIC as low as 20 μ g/ml as also been reported against *C.albicans*[27][28]. The motility inhibition assay displayed that the motility of the test microbes was affected. The cinnamic acid and flavonoids component present in Propolis are known to inhibit the motility by uncoupling the energy transducing cytoplasmic membrane [29]. The current study showed that the North Indian Propolis has antimicrobial activity against Gram positive organism *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC2470), Gram negative *Escherichia coli* (MTCC 47), *Enterobacter cloacae* (MTCC 9145), *Pseudomonas aeruginosa* (MTCC 424) and fungus *Candida albicans* (MTCC 227). The motility of the test microorganisms was also affected. The results are in agreement with the earlier studies done with Propolis from different parts of the world.

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