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**Original Article**

# The Prevention and Treatment of *Staphylococcus aureus* Biofilm Formation using Russian Propolis Ethanol Extracts

Julia Bryan[1], Tung Po Chen[2], Tianchi Liu[3], Robin Azzam[3], Konstantinos N. Belonis[1],Michael Castaldi[1], Jessica Epstein[1], and Christian Traba[1]*

Department of Chemistry, Saint Peter's University, Jersey City, NJ 07306, USA[1], Department of Civil, Environmental and Ocean Engineering, Charles V. Schaefer School of Engineering and Sciences, Stevens Institute of Technology, Hoboken, NJ 07030, USA[2] and Department of Chemistry, Chemical Biology, and Biomedical Engineering, Charles V. Schaefer School of Engineering and Sciences, Stevens Institute of Technology, Hoboken, NJ 07030, USA[3]

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## ABSTRACT

In this study, the exposure of three Russian Propolis ethanol extracts from different regions in Russia was used in: 1) the prevention and 2) treatment of *Staphylococcus aureus* biofilms on various surfaces. It was found that despite similar activities against biofilm prevention (planktonic bacteria), drastic differences were observed in biofilm treatments. The antibacterial and anti-biofilm results indicated that: 1) antibacterial and 2) anti-biofilm activity of Russian propolis ethanol extracts may be governed by different types of chemical compounds found in the propolis ethanol extracts. Further analysis indicated that the mechanism of action of propolis ethanol extracts appear to be complex and involves the degradation of the extracellular polymeric matrix, exposing the bacteria within the biofilm to the antibacterial agents found in the propolis extracts. Kinetic studies conducted under optimal experimental conditions, revealed that bacterial cells in *Staphylococcus aureus* biofilms were killed (>99.9%) by propolis after 12 hours of treatment. The sterilization ability of propolis for the prevention and treatment of biofilm related contaminations on various materials were confirmed and information gathered from this study provides insights into the anti-biofilm mechanism of propolis.

**Keywords:** Propolis ethanol extracts; decontamination; sterilization; anti-biofilm; *Staphylococcus aureus* biofilms.

**Abbreviations:** MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, ATCC: American Type Culture Collection, TSBG: Tryptic soy broth supplemented with 0.2% glucose, CV: Crystal Violet, PBS: phosphate buffered saline, *S. aureus: Staphylococcus aureus*, SEM: scanning electron microscopy MIC: minimal inhibition concentration, SDS: sodium dodecyl-sulfate.

## INTRODUCTION

The adhesion of bacteria to a surface is mediated by different types of interactions, which can be specific, such as protein film formation or nonspecific interactions such as surface energy, surface charge, hydrophobicity and van der Waals forces.[1,2] Once adhered to a surface, the bacteria form communities which develop into biofilms and this serves as reservoirs for the development of pathogenic infections.[3,4] *Staphylococcus aureus*, an example of just one theses pathogenic infections, can grow biofilms with thicknesses of up to 20 µm after 24 hours of incubation[5] and account for the majority (>70%) of hospital acquired infections. These bacterial biofilms are often associated with long periods of hospitalization, morbidity and death.[6,7]

During biofilm formation, an extracellular polymeric matrix is produced and plays significant roles in both the structure and function of biofilm communities. The formation of the extracellular polymeric matrix provides considerable advantages such as: protection against antimicrobial agents, acquisition of new genetic traits, nutrient availability and metabolic cooperability.[8] The biofilm architecture serves as a physical barrier to

***Corresponding Author Address:** Dr. Christian Traba, Department of Chemistry, Saint Peter's University, 2641 John F. Kennedy Boulevard, Jersey City, NJ 07306, USA; E-mail: ctraba@saintpeters.edu*

antimicrobial agents as well as the hosts immune system, by blocking access to the bacteria embedded deep within the biofilm.[9] As a result, these bacterial communities are up to 1000x's more resistant to antibiotics and the hosts immune system in comparison to planktonic bacteria.[10,11]

In response to the resistant and infectious attributes of biofilms, propolis, a known antibacterial, predated only by the discovery of honey[12], was investigated for the decontamination of mature *S. aureus* biofilms. Although many types of propolis extracts originating from different regions of the world have been proven effective against a wide range of free-living microorganisms, only a handful of studies have been carried out to determine the inhibitory effect of propolis against bacteria of within mature biofilms and an even fewer number have considered the mechanism of action. Propolis, a natural product derived from plant resins, is a strongly adhesive, resinous substance collected and transformed by bees. Honeybees use propolis for the construction and maintenance of their hives walls in order to protect entrance from intruders.[13] This multifunctional substance contains a complex mixture of chemical constituents. The chemical composition of propolis is dictated by the constituents of the plant material making up the native vegetation as well as the season of collection.[14] Propolis, is reported to contain more than 210 different compounds including, aliphatic acids, aromatic esters and acids, flavonoids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, vitamins, minerals and enzymes including succinic dehydrogenase, glucose- 6-phosphatasechalcones, which are all reported to play significant roles in the antibacterial activity of propolis.[15-19]

Propolis, is one of the few natural remedies that has maintained its popularity over a long period of time. In addition to its antibacterial activity, propolis ethanol extracts have also been tested and confirmed for several other biological activities including anti-inflammatory, tissue regenerative, wound healing and anti-carcinogenic, etc.[20-24] The objectives of this study are to: 1) expand on the potential application of propolis ethanol extracts not only against gram-positive *S. aureus* planktonic bacteria but also towards bacteria embedded within the protective coating of mature *S. aureus* biofilms and 2) begin to unravel the mechanism of action of propolis ethanol extracts for the sterilization of *S. aureus* biofilm contaminated surfaces.

## MATERIALS AND METHODS

**Bacterial strains and medium:** *Staphylococcus aureus* (penicillin resistant, ATCC 29213, penicillin sensitive, 25923, and methicillin resistant, ATCC 43300), all good biofilm forming *S. aureus* strains, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). We grew *staphylococcus* bacteria in tryptic soy broth (TSB) supplemented with 0.2% glucose (TSBG). For each experiment, an isolated single bacterial colony was picked from a tryptic soy broth agar plate, transferred to 10–15 ml of medium, and then incubated under orbital agitation (100–150 rpm) at 37 ºC for 18–24 h.

**Reagents and solutions:** A LIVE/DEAD staining kit was purchased from Invitrogen Life Technologies (Carlsbad, CA) for the staining of bacteria within biofilms. Also, 5% methyl thiazolyl diphenyl-tetrazolium bromide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), in phosphate buffered saline (PBS), crystal violet (CV), sodium dodecyl-sulfate (SDS) and other reagents were all purchased from the Sigma Chemical Laboratory (St Louis, MO).

**Growth of biofilms on different materials:** For each experiment, an isolated single bacterial colony was picked from an agar plate, transferred to 10–15 ml of TSBG medium and then incubated under orbital agitation (100–150 rpm) at 37 ºC for 18–24 h. This overnight culture of *S. aureus* was diluted in TSBG to $2\times10^6$ cells $ml^{-1}$ and then inoculated on surfaces of different materials including 8-well glass chambers, polyethylene terephthalate films, polystyrene 6-well plates, and silicon wafers. *S. aureus* biofilms of 15~20 µm in thickness were formed on all tested materials within 24 hrs, were used throughout this work.

**Preparation of ethanolic extracts of propolis:**
Propolis samples were gathered from three regions of Russia: Crude propolis sample I was obtained from Krasnodar Krai region of Russia, which is located in the southwestern part of the North Caucasus. Crude propolis samples II and III were obtained from apiaries of the Ural Mountains region, East European part of Russia. Hand collected propolis were kept desiccated and in the dark up to their processing. Propolis samples were ground with minimal amounts of ethanol with continuous stirring at room temperature for 30 days. The suspension was filtered and then centrifuged at 3000 rpm for 20 min to remove all un-dissolved propolis. The supernatant was then concentrated in a rotary evaporator under reduced pressure at 40 ºC and re-dissolved (1 ml of 99 % ethanol was used to dissolve 0.3 mg of propolis) and kept at room temperature in the dark until use (Santos et al. 1999).

**Planktonic bacterial and biofilm assays**: It is known that planktonic bacteria once adhered to a

surface form biofilms. The purpose of this experiment was to test the ability of the propolis ethanol extracts to prevent biofilm formation. In this assay, 4 mL of midlogarithmic (mid-log) phase bacteria (~ $2 \times 10^6$ cells/ml) suspended in TSBG medium were exposed to propolis ethanol extracts with volumes ranging from 0 to .100 mL (0 to 20 µg) for 24 h at an incubation temperature of 37 $^oC$. After the 24 hours of incubation, the formation of biofilms was verified using the popular CV staining method in combination with the MTT based viability assay to assess biofilm susceptibility to the propolis ethanol extracts. Unlike CV staining, which is used for staining bacterial cells (both live and dead) and other macromolecules such as polysaccharides, DNA and proteins in the biofilm extracellular matrix, the MTT assay was designed for live bacteria by measuring the overall metabolic activity of bacterial cells in biofilms. Thus, CV staining was used for the quantification of biofilms (total biomass of biofilm) while the MTT assay staining was utilized to evaluate viability of bacteria in biofilms and DNA/polysaccharides in the biofilm extracellular matrix. It should be stated that there is an excellent correlation between formazan concentrations (absorbance at OD 570 nm) and CFU counting.

In CV staining, biofilms were stained with 0.1% (w/v) CV for 10 min. The excess dye was removed by thoroughly rinsing the plate with water. CV dye associated with biofilms was then extracted by 33% glacial acetic acid and quantified using a microplate reader by measuring solution absorbance values at 570 nm. The CV and MTT assays were also used for pre-formed biofilms as well.

In the MTT assay, biofilms were incubated with MTT at 37 ºC for 30 min. After washing, the purple formazan formed inside the bacterial cells was dissolved by SDS and then measured using a microplate reader by setting the detecting and reference wavelengths at 570 nm and 630 nm, respectively.

**Antibacterial activity of studies of ethanol extracts of propolis:** At the end of incubation, the formed biofilms were washed with PBS in order to remove planktonic and loosely attached bacteria. *S. aureus* biofilms of 15~300 µm in thickness were formed on all tested materials after seven days of incubation. Pre-formed biofilms on various substrates were washed with PBS to remove loosely bound bacteria. All ethanol extracts of propolis were weighed under aseptic conditions in sterile volumetric flasks and different volumes 0 to 400 µL (0 µg- 120 µg) were pipetted into fresh 4 mL of TSBG. These TBSG solutions containing the different amounts of propolis ethanol extract were used to treat both planktonic bacteria and pre-formed biofilms. After exposure to propolis extracts for up to 24 hours, TSBG containing propolis was removed and the biofilms were once again washes with PBS.

**Staining of live and dead bacteria:** Live and dead bacterial distributions of planktonic bacteria as well as bacteria in biofilms were studied by confocal laser scanning microscopy using a LIVE/DEAD staining kit as described previously.[8] Biofilms grown on LabTek 8-well cover-glass chambers were washed with PBS to remove planktonic bacteria and TSBG medium. After that LIVE/DEAD dyes in PBS were added and incubated for 15 minutes at room temperature. Stained live (green) and dead (red) bacteria in biofilms were visualized by confocal fluorescence microscopy according to the protocol provided by the manufacture.

**Scanning electron microscopy:** SEM images were obtained using an Auriga scanning electron microscope. Silicon wafers containing adhered *S. aureus bacteria* were treated with argon plasma under specific experimental conditions. These samples were attached to the SEM stage using conductive tape and coated with gold for 30 sec. The applied voltage was 2 kV.

## RESULTS AND DISCUSSION

**Antibacterial activity of Russian propolis ethanol extracts:** The composition of propolis depends mainly on the vegetation at the site of collection. Due to differences in the geographical locations among the propolis samples used in this study, variances in the chemical compositions are expected and therefore, ethanol extracts from each sample of propolis was expected to demonstrate different antibacterial activity. However, that was not the case with respect to planktonic bacteria (Fig. 1A). In this study, the antibacterial activity of propolis ethanol extracts was observed through two significantly different assays; 1) viability (MTT) and 2) biomass (CV). Each propolis ethanol extract (I, II, or III) showed similar and very potent antibacterial activity towards *S. aureus* planktonic bacteria. The viability of *S. aureus* planktonic bacteria decreased with respect to propolis ethanol extracts concentration. Up until 3 µg of propolis, significant numbers of bacteria were still viable (Fig. 1A). Beyond 4µg of propolis ethanol extract, complete planktonic bacterial death is observed (Fig. 1A). Based on the results, it seems reasonable that the antibacterial activity may stem from common chemicals compounds present in each of the three propolis ethanol extracts, suggesting that

the antibacterial activity may be caused by some synergistic effects of propolis, which is in agreement with outside studies.[25,26]

Viable planktonic bacteria results in the colonization of a surface and eventually in the formation of biofilms, the CV assay was another method used in evaluating the efficiency and effectiveness of these Russian propolis ethanol extracts against planktonic bacteria by monitoring the formation of biofilms. Biofilm formation (CV assay) was proportional to the numbers of viable bacteria (MTT assay), indicated by high absorbance readings. On the other hand, complete bacterial inactivation was confirmed by the absence of bacterial biofilms found in samples treated with >4µg of propolis (Fig. 1B).

In addition to the MTT method, another cell viability assay using a Live/Dead staining kit was used to evaluate the antibacterial activity of the different propolis samples. The SYTO 9 dye, a nucleic acid probe with green fluorescence color, is permeable to healthy cell membranes and thus bacteria with intact (live) cell membranes are stained green. Propidium iodide, a nucleic acid probe with red fluorescence color, is cell membrane impermeable and thus only stains dead bacteria with damaged cell membranes. Confocal microscopy images of treated planktonic bacteria are consistent with the results obtained in the MTT assay and confirm that the antibacterial activity of propolis towards *S. aureus* cells is concentration dependent (Fig. 1C). In response to the significant activity of propolis, we chose to expand on this study and test these same three propolis ethanol extracts against mature *Staphylococcus aureus* biofilms. *S. aureus* biofilms had normal architectural components with proven resistance to antibiotic treatment.[27]

**Anti-biofilm activity of Russian propolis ethanol extracts:** Although the antibacterial activity of propolis has already been demonstrated against Gram-positive strains and eventually confirmed in this study, the aim of the present work was to evaluate the activity of these propolis samples against various strains of *S. aureus* bacterial biofilms. Since very few reports have used propolis for the decontamination of bacteria within mature biofilms, good biofilm forming *S. aureus* strains (ATCC 29213, ATCC 25923, and ATCC 43000) were cultured and their susceptibility to propolis was evaluated. These *S. aureus* strains of bacteria can grow into biofilms of 15–300 µm thickness in TSBG, depending on duration of incubation. Mature biofilms (1 to 7-days old), were cultured specifically and on various materials including six-well plates, 8-well glass chambers and silicon wafers, depending on the experimental specifications and microscopy requirements.

In these mature biofilms, individual bacteria were protected and attached to one another by an extracellular polymeric substance, which is secreted by the bacteria and indicated by red arrows (Fig. 2). The idea behind exposing propolis to biofilms was to test the antibacterial activity of propolis against the resistance nature commonly associated with these biofilms. Although there was slight pH drop from 7.4 – 6.8, after the addition of propolis ethanol extracts to TSBG culture media, the pH change had no measurable effect on the activity of the TSBG solution containing propolis, as determined by changing the pH of TSBG to 6.8 and exposing this culture media to the bacteria as well as biofilms (data not shown). Throughout this study, *S. aureus* planktonic bacteria as well as *S. aureus* biofilms, were subjected to TSBG culture media containing exact volumes of 99% pure ethanol (0 mL to .400 mL), which was used for the extraction of propolis. In these control groups, almost 98% of *S. aureus* planktonic bacteria and the *S. aureus* bacteria in biofilms were still alive (data not shown). These results confirm that the antibacterial/anti-biofilm activity of propolis was dependent solely on the various chemical compositions associated with each sample of propolis ethanol extract. Results from biofilm studies suggest that the extracellular polymeric matrix could protect (to a certain extent) these infectious microorganisms from the antibacterial chemicals found in propolis ethanol extracts as it regularly does against the host's immune system and antibiotic attack. Since the biofilm acts as a barrier preventing the active compounds in propolis from inactivating *S. aureus* bacteria in biofilms, higher propolis concentrations in comparison to the amount of propoplis required to kill planktonic *S. aureus* bacteria is needed. These results indicate that the extracellular polymeric matrix could limit the exposure of the active chemicals found in propolis to the bacteria, allowing bacteria to thrive under higher (<10 µg) concentrations of propolis. Up until 9µg of propolis I, there is a gradual decline in the numbers of viable *S. aureus* bacteria in biofilms, but the bacteria embedded deep within the biofilm thrived (Fig. 3A). However, at propolis concentrations above 10µg (MIC), complete biofilm inactivation were achieved in propolis I ethanol extracts (Fig. 3A). The same was not the case for biofilms exposed to propolis samples II and III. In fact, decreased killing activities were noticed for these propolis ethanol extracts even at higher concentrations of propolis (>30µg). Interestingly, as the numbers of viable bacteria were reduced in biofilms treated with propolis I, significant decreases in biomass were observed

(Fig. 3B). It was not surprising that propolis ethanol extracts from crude samples II and III showed similar results since they were taken from the areas of close proximity to one another. As a result, these crude samples possibly contain nearly identical chemical compositions and therefor, show nearly identical activities.

**Activities of the three Russian Propolis extracts:** It was very surprising to see that the activities of propolis samples against *S. aureus* biofilms varied drastically and were not consistent with antibacterial activity assays. Bacterial and biofilm inactivation with propolis is concentration dependent. Ethanol extracts from propolis I showed more potent anti-biofilm activity in comparison to Propolis II and Propolis III samples as demonstrated in MTT assays (Fig.3A). A reasonable explanation may be that the antibacterial and anti-biofilm activities of propolis ethanol extracts stem from different chemicals or combinations of chemicals. Therefore, place of origin may create variation and concentration differences among the chemical compositions of propolis and this may affect the anti-biofilm properties. It should be stated that the purpose of this work was not to determine the active compounds in propolis. Establishing between the chemicals or compounds involved in either the antibacterial or anti-biofilm activity is a future direction of this project. With that said, it is possible that: 1) ethanol extracts from propolis II and III contain similar antibacterial compounds but different anti-biofilm compounds; or 2) propolis ethanol extracts from II and III possess both the same antibacterial and anti-biofilm compounds as in propolis I but in significantly less amounts with respect to anti-biofilm compounds. At particular high (>20 µg) concentrations of propolis II and III used in this study, limited activity towards *S. aureus* bacteria within biofilms was observed and reached a plateau. At these concentrations, diffusion of active compounds through the biofilm was limited. The active compounds could not reach the bacteria deep within the protective surroundings of the extracellular matrix and interact specifically with the bacteria deep within the confinements of the biofilm to cause bacterial cell death.

**Anti-biofilm mechanism studies of Russian propolis extracts:** Based on the results acquired in Figure 3, propolis I ethanol extracts were focused on due to its potent anti-biofilm activity over the other propolis extracts. The activity of propolis I ethanol extracts against different strains of *S. aureus* bacterial biofilms was studied and the results were not surprising. As already seen in anti-biofilm *S. aureus* (29213) studies, (Figure 3), propolis I ethanol extracts were effective towards both *S. aureus* (43300) and (25923) strains of bacteria (Fig. 4). In these experiments, similar killing curves were demonstrated and the activity of propolis I ethanol extracts towards different strains of *S. aureus* biofilms is confirmed (Fig. 4A). We know that some chemicals found in propolis extracts cause bacterial cell death. The question now becomes: 1) how do these antibacterial compounds gain access to the bacteria deep within the biofilm? This question was studied through Z-stack confocal microscope imaging technology of biofilms, which provided an opportunity to visualize and quantify the contributions of the anti-biofilm activity ability of propolis treated samples along with corresponding biomass staining assays.

Preliminary data for the proposed mechanism of action was obtained in Figure 3 and suggests that the anti-biofilm mechanism can be attributed to the chemical degradation ability of propolis with respect to the biofilm's extracellular polymeric matrix. If biofilms are inactivated through this mechanism, the CV staining results should indicate biofilm biomass loss with respect to increased killing activity. Data obtained from Figure 3b shows that decreases in biomass accompanies increased killing activities (indicated with green arrow in Fig. 3B). On the other hand, significant biomass removal was not present in biofilms treated with either propolis II or III ethanol extracts (indicated with red arrow in Fig. 3B) and biofilms treated with these extracts did not inactivate bacteria to the extent that propolis I did (Fig. 3A). Only at high concentrations is significant activity for these propolis II and III samples achieved but still do not achieve the same killing activity at propolis I. These results indicate that propolis II and III extracts possess both the antibacterial properties and the anti-biofilm compounds but in much lower concentrations with respect to the anti-biofilm compounds. In order to test the proposed mechanism of action, the inactivation of biofilms using minimal inhibition concentration (20 µg) at different exposure times was studied. In these kinetic studies, the anti-biofilm activity was a gradual process, which took 12 hours to achieve >99.9% killing (Fig. 5A). CV assays confirm that reductions of live bacteria are proportional to biomass loss (Fig. 5B). Although longer exposure times were associated with improved effectiveness of propolis ethanol extracts in killing bacteria in 1-day old biofilms, treatment times beyond 12 hours did not result in improved activity (>99.9%). Corresponding Live/Dead confocal microscopy images were taken and the results match nicely with both MTT and CV results (Fig. 4C). Z-stack confocal microscope images show the reductions in biofilms but with respect to biofilm thickness as

treatments times are increased, confirming the removal ability of propolis (Fig. 5C). Untreated 1-day old *S. aureus* biofilms were about 18 µm in thickness and were filled with live bacteria (stained green). The thickness of the same biofilm gradually reduced with treatment time and was finally reduced to 4-6 µm after treatment with 20 µg of propolis for 12 hours. The results obtained in this study indicate that as the numbers of viable bacteria are decreased, reduction in the biomass of the same treated biofilms is observed. It is believed that as the chemical and structural integrity of the extracellular polymeric matrix becomes compromised by the anti-biofilm compounds in the propolis ethanol extracts. As a result, the polymeric coating is no longer able to provide protection to the bacteria. The inability of the biofilm to protect the infectious microorganism allows access of the antibacterial chemicals to the bacteria. Eventually, the bacteria succumb to the antibacterial agents. As the damage becomes more severe to the extracellular polymermic matrix, removal of the coating from the surface of the biofilm occurs. Consequently, the bacteria that were once surrounded by the protective coating of the biofilm are now exposed to the antibacterial properties of the propolis and reduction in thickness occurs, indicated by red arrows (Fig. 5C). In order to test the proposed acting mechanism of propolis ethanol extracts, 7-day old biofilms were grown. SEM images show that aged biofilms contain more extracellular polymeric matrix and have more sophisticated biofilm structures (Fig.2B) and is significantly different from what was observed for 1-day old biofilms (Fig. 2A). It is expected that the more sophisticated biofilm structures with drastic increases in biofilm thickness would play an even more integral role in protecting the bacteria deep within the biofilm against the anti-biofilm activity of propolis ethanol extracts. For this reason, the anti-biofilm activity of propolis ethanol extracts was tested on 7-day old biofilms. Unlike the 1-day old biofilms, *S. aureus* cells in 7-day old biofilms were completely buried inside the extracellular polymeric matrix and very few single bacteria could be identified using SEM (Fig. 2B). MTT (Fig. 6A) along with confocal microscopy images (Fig. 6C) of 7-day old biofilms, show that only <30% of *S. aureus* cells in 7-day old biofilms were killed after being exposed to 20 µg of propolis as compared to 1-day old biofilms. Bacteria that were wrapped inside these biofilm structures with a rich extracellular polymeric matrix were protected and consequently more difficult to treat than 1-day old biofilms, which were susceptible to 20 µg of the propolis ethanol extract. Exposure concentrations of 40µg required more than 18 hours to substantially reduce the number of viable bacteria in the 7-day old biofilms and were accompanied by a reduction in biomass along with biofilm thickness. In order to achieve significant (>99.9%) inactivation, an exposure concentration of 120 µg for up to 24 hours was required and involved a drastic reduction in biomass (>60%). Obviously, the impact of the degradation effect on the anti-biofilm activity of propolis is influenced greatly by the maturation of biofilms (Fig. 6B). As z-stack confocal microscopy images are considered, reductions of biofilm thicknesses (300 µm to <100 µm) was observed as the numbers of live bacteria were decreased after optimal exposure of propolis was achieved, confirming that complete decontamination of biofilms is achieved through the chemical degradation of biofilm architectural components.

## CONCLUSION

Unlike many “natural” remedies, there is a substantial database on the biological activity and toxicity of propolis indicating it’s many pharmaceutical properties including antibiotic, antifungal, antiviral and antitumor, among other attributes.[28] Although many groups have studied the activity of propolis ethanol extracts against planktonic bacteria, the activity of propolis ethanol extracts have rarely been studied against bacteria protected within biofilms. The results discussed in this study begin to unravel the mechanisms of action of propolis ethanol extracts and provide evidence for the successful decontamination of *S. aureus* biofilms. Our results present unambiguous proof that propolis samples from various regions of Russia have similar antibacterial activities but drastic differences in anti-biofilm activities. Such differences are due to the chemical compositions of propolis samples, stemming from different geographic locations. In this study, the mechanism of action of propolis ethanol extracts appears to be complex and involves the degradation of the extracellular polymeric matrix, which affected the architectural and chemical integrity of the biofilm causing the biofilm to become unable to protect the bacteria and eventually exposing the bacteria to the antibacterial agents of the propolis. Our results, as well as the literature data dealing with chemical composition cannot point out one individual substance or a particular chemical class, which could be responsible for this action. Obviously, in different samples, different concentrations of chemicals and/or chemical combinations are present and affect the anti-biofilm activity. Future studies involving the linking of chemicals to particular activities will be conducted.

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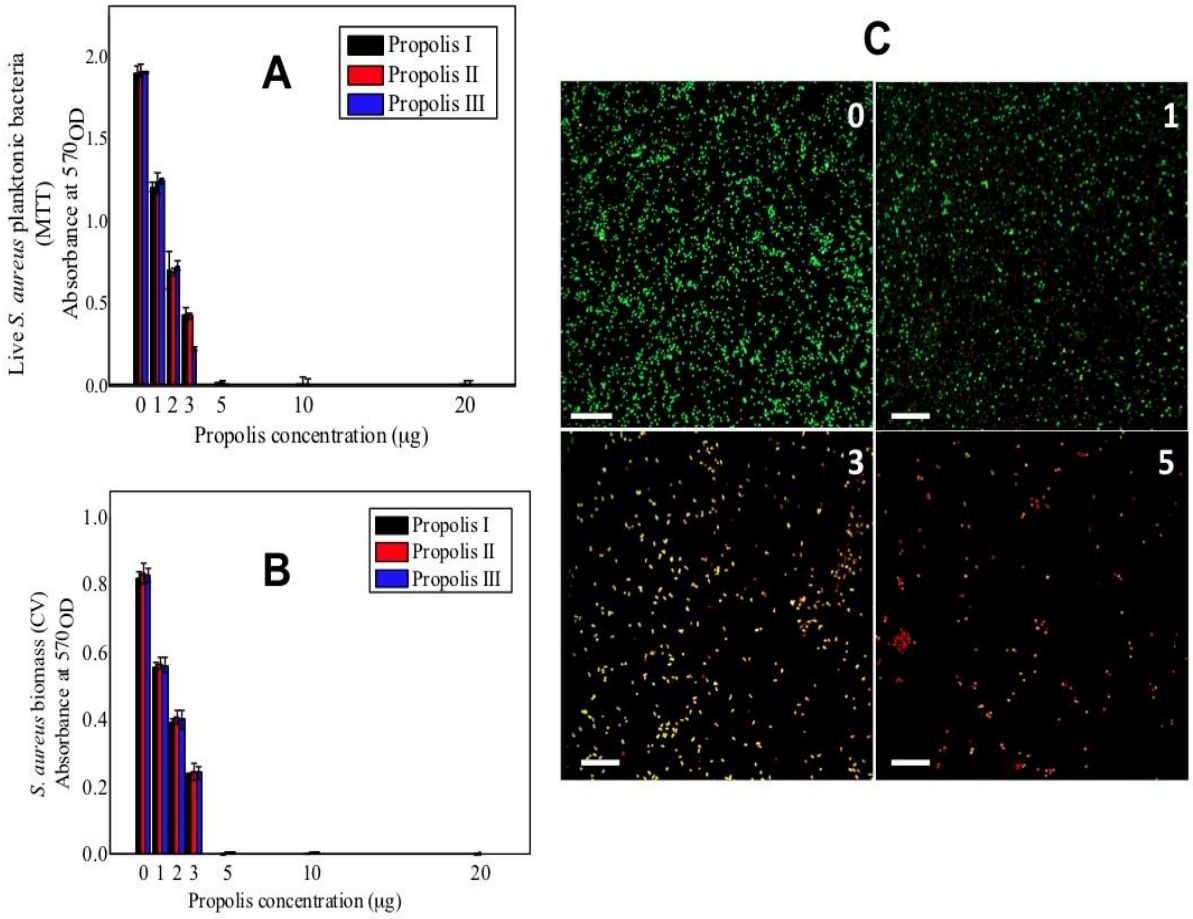


Figure 1. Propolis ethanol extracts caused planktonic bacteria death. Bacterial viability (A) and biofilm formation (B) were determined by MTT and CV staining assays, respectively. Planktonic *S. aureus* (29213) bacteria were treated with 0 – 20 μg of the three different samples of propolis extracts for 24 hours. Viable bacterial numbers and biofilm formation were quantified by measuring absorbance changes in biofilms at OD570 nm after treatment. Data represents the mean and SD of at least three samples. Fluorescence confocal microscopy images of planktonic bacteria treated with propolis I ethanol extracts of concentrations 0 μg ($C_0$); 1 μg ($C_1$); 3 μg ($C_3$); and 5 μg ($C_5$) were taken. Bacteria were stained with Live/Dead staining kit. Live bacteria were stained green and dead bacteria were stained red, respectively. Scale bar = 20.0 μm.

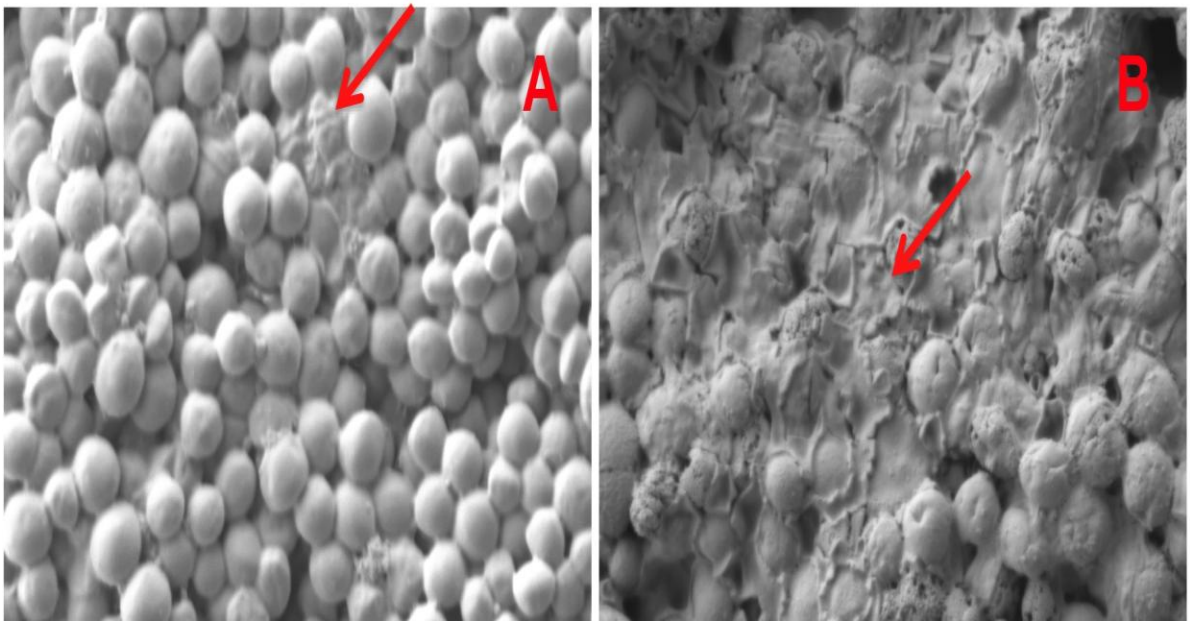


Figure 2. SEM image of mature one-day old (A) and seven-day old (B) *S. aureus* (29213) biofilms. Scale bar = 1.0 μm.

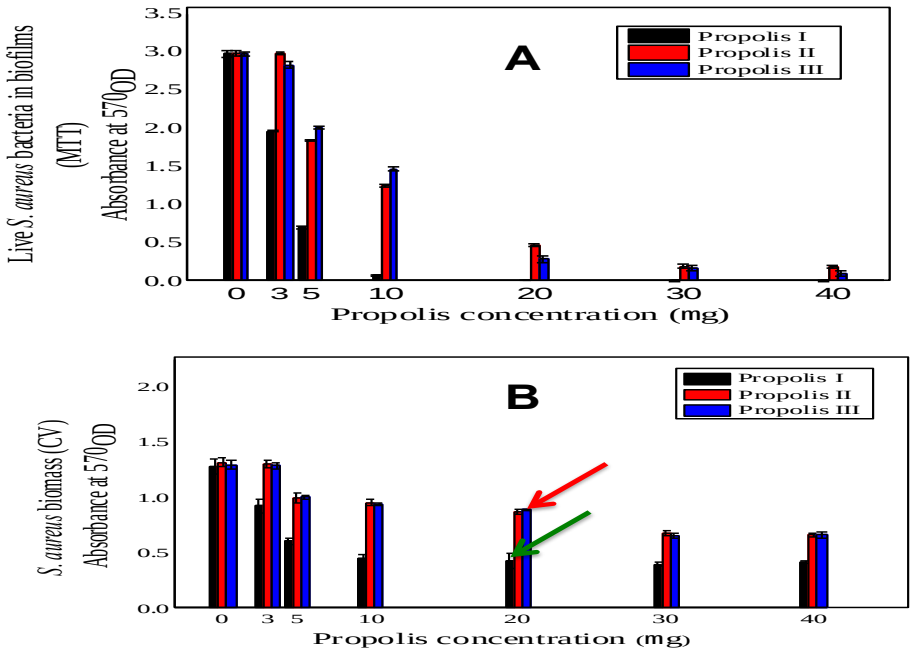


Figure 3. Propolis ethanol extracts caused bacterial death and biomass loss in pre-formed 1-day old *S. aureus* (29213) biofilms grown on 6-well plates, after treatment for 24 hours. Bacterial viability (A) and biomass loss (B) were determined by MTT and CV staining assays, respectively. Viable bacterial numbers and biomass loss were quantified by measuring absorbance changes in biofilms at OD570 nm after treatment. Data represents the mean and SD of at least three samples.

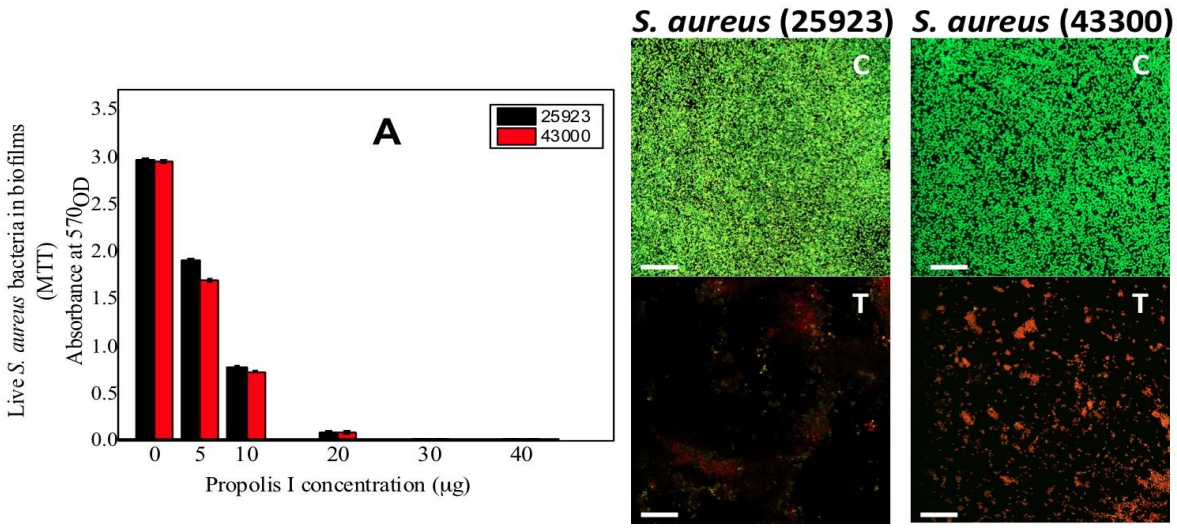


Figure 4. Propolis ethanol extracts caused bacterial death in pre-formed 1-day old *S. aureus* (25923 and 43300) biofilms grown on 6-well plates, after treatment for 24 hours. Bacterial viability (A) along with corresponding Fluorescence confocal microscopy images of propolis I ethanol extract mediated biofilm inactivation of biofilms grown on PET films. Viable bacterial numbers were quantified by measuring absorbance changes in biofilms at OD570 nm after treatment. Data represents the mean and SD of at least three samples. Control groups (C), represent biofilms not exposed to any propolis extracts, while treated groups (T), represent biofilms treated with 20 µg of propolis ethanol extract for 24 hours. Bacteria in biofilms were stained with Live/Dead staining kit. Live bacteria were stained green and dead bacteria were stained red, respectively. Scale bar = 20.0 µm.

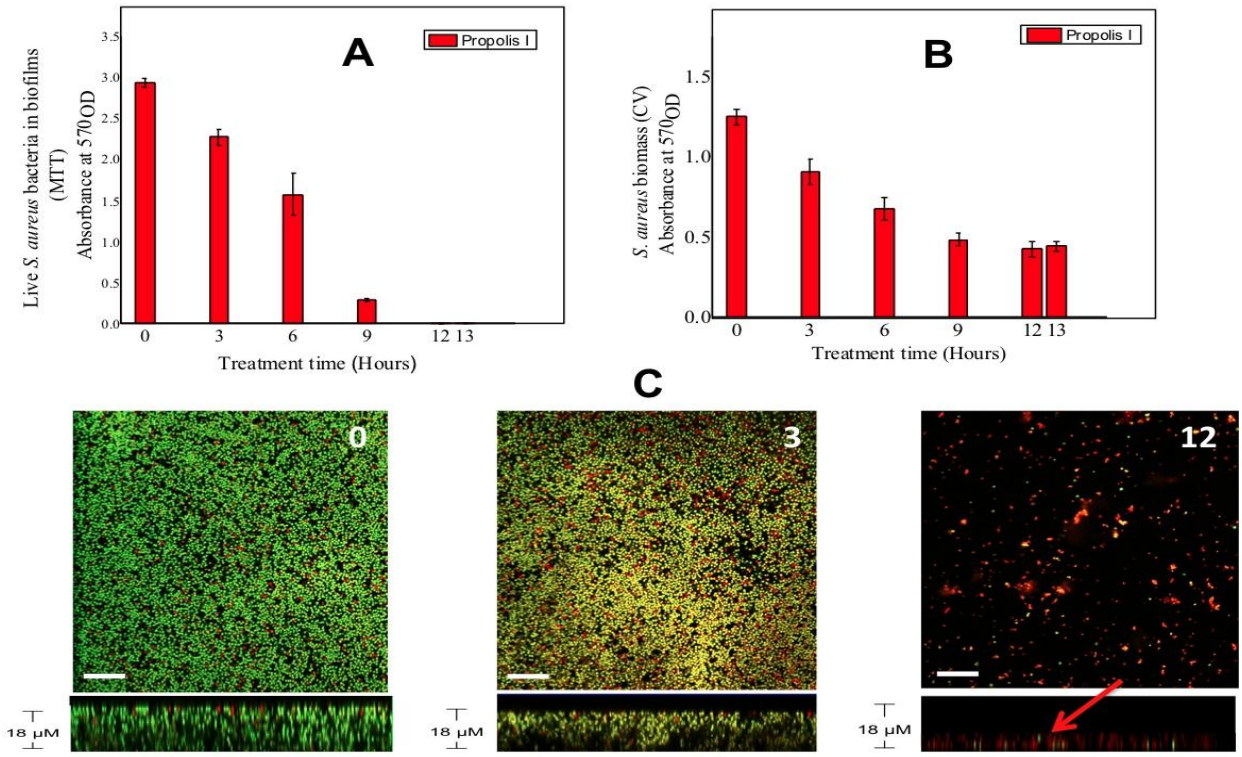


Figure 5. Biofilms were treated with 20 µg of propolis ethanol extracts for 13 hours. Different time points were selected and bacterial death and biomass loss in pre-formed 1-day old *S. aureus* (29213) biofilms grown on 6-well plates was monitored. Bacterial viability (A) and biomass loss (B) were determined by MTT and CV staining assays, respectively. Bacterial numbers and biomass loss were quantified by measuring absorbance changes in biofilms at OD570 nm after treatment. Data represents the mean and SD of at least three samples. Fluorescence confocal microscopy images along with corresponding z-stack images of propolis I ethanol extract mediated biofilm inactivation and biomass removal of *S. aureus* biofilms grown on PET films at treatment times of 0 hour ($C_0$); 3 hours ($C_3$); and 12 hour ($C_{12}$) time points were taken. Bacteria were stained with Live/Dead staining kit. Live bacteria were stained green and dead bacteria were stained red, respectively. Scale bar = 20.0 µm.

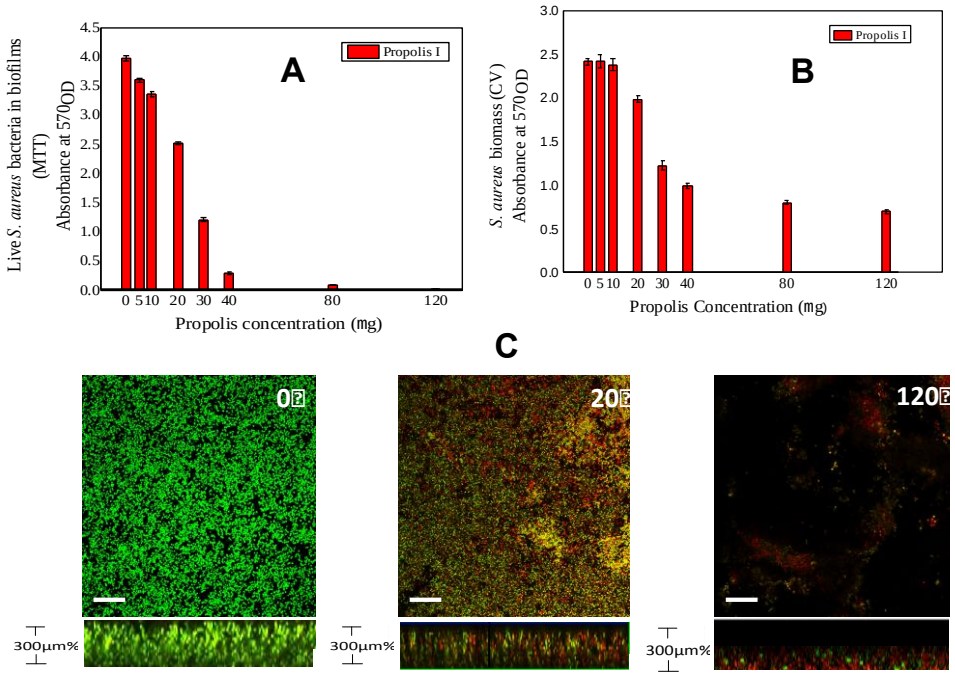


Figure 6. Propolis ethanol extracts caused bacterial death and biomass loss in pre-formed 7-day old *S. aureus* (29213) biofilms grown on 6-well plates. Bacterial viability (A) and biomass loss (B) were determined by MTT and CV staining assays, respectively. Bacterial numbers and biomass loss were quantified by measuring absorbance changes in biofilms at OD570 nm after treatment. Data represents the mean and SD of at least three samples. Fluorescence confocal microscopy images along with corresponding z-stack images of propolis I ethanol extract mediated biofilm inactivation and biomass removal of 7-day old *S. aureus* (29213) biofilms grown on PET films treated with 0 µg ($C_0$); 20 µg ($C_3$); and 120 µg ($C_{120}$) of propolis I ethanol extracts were taken. Bacteria were stained with Live/Dead staining kit. Live bacteria were stained green and dead bacteria were stained red, respectively. Scale bar = 20.0 µm.

## References


1. Cos P., Tote K., Horemans T., and Maes L.. Biofilms: an extra hurdle for effective antimicrobial therapy. Current Pharmaceutical Design, 2010; 16: 2279-2295.
2. Nagel JA., Dickinson RB., and Cooper SL.. Bacterial adhesion to polyurethane surfaces in the presence of pre-absorbed high molecular weight kinnogen. Journal of Biomaterial Science Polymer Education, 1996; 7: 769-780.
3. Costerton JW., Lewandowski Z., Caldwell DE., Korber DR., and Lappin-Scott HM. Microbial biofilms. Annual Review Microbiology, 1995; 49: 711-745.
4. Bazaka K1., Jacob MV., Crawford RJ., and Ivanova EP. Plasma-assisted surface modification of organic biopolymers to prevent bacterial attachment. Acta Biomaterialia, 2011; 7: 2015–2028.
5. Traba C., Chen L., Azzam R., Liang JF.. Insights into discharge argon mediated biofilm inactivation. Biofouling, 2013; 29: 10: 1205-1213.
6. Darouiche RO. Treatment of infections associated with surgical implants. New England Journal of Medicine, 2004; 350:1422e9.
7. Mah TFC., and O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiology, 2001; 2001: 9:34e9.
8. Traba C., and Liang JF. Susceptibility of Staphylococcus aureus biofilms to reactive discharge gases.Biofouling, 2011; 27: 7: 763-772.
9. Willcox MD., Hume EB., Aliwarga Y., Kumar N., *et al.* A novel cationic-peptide coating for the prevention of microbial colonization on contact lenses. Journal Applied Microbiology, 2008; 105: 6: 1817–1825.
10. Costerton JW., Stewart, PS., and Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science,1999;284: 1318–1322.
11. Donlan RM., and Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology, 2002;15: 167–193.
12. Santos FA., Iorio NLP., Lobo LA., dos Santos K., *et al.* Antibacterial activity of propolis produced in Brazil against Actino bacillus action mycetemcomitans, Fusc bacterium spp. and bacteria from Bacteroides Fragilis group isolated from human and marmoset hosts. Anaerobe, 1999; 5: 479-481.
13. Park YK., Ikegaki M., and Alencar SM. Classification of Brazilian propolis by physicochemical method and biological activity. Mensagem Doce, 2000; 58: 2-7.
14. Bankova V., Castro SL., and Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie, 2000; 31: 3-15.

15. Chang R., Veloso D., Morais S., Nascimento A. *et al*. Analysis of a Brazilian green propolis from Baccharis dracunculifolia by HPLC-APCI-MS and GC-MS. Brazilian Journal of Pharmacognosy, 2008; 18: 549-556.
16. Daugsch A., Moraes CS., Fort P., and Park YK. Brazilian red propolis-chemical composition and botanical origin. Evidence Based Complementary Alternative Medicine, 2008; 5: 435-441.
17. Majiene D., Trumbeckaite S., Grūnoviene D., Ivanauskas L., *et al*. Investigation of chemical composition of propolis extract. Medicina (Kaunas), 2004; 40: 771-774.
18. Sousa JPB., Furtado NAJC., Jorge R., Soares AEE., *et al*. Physical chemical and chromatographic profiles of propolis samples produced in the micro regions of Franca (SP) and Passos (MG), Brazil. Brazilian Journal of Pharmacognosy, 2007; 17: 85-93.
19. Teixeira EW., Message D., Negri G., Salatino A., *et al*. Seasonal variation, chemical composition and antioxidant activity of Brazilian propolis samples. Evidence Based Complementary Alternative Medicine, 2008; 7: 307-315.
20. Chen CN., Weng MH., Wu CL., and Lin JK. Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources. Evidence Based Complementary Alternative Medicine, 2004; 1: 175-185.
21. Duran N., Koc A., Oksuz H., Tamer C., *et al*. The protective role of topical própolis on experimental keratitis via nitric oxide levels in rabbits. Molecular Cell Biochemistry, 2006; 281: 153-161.
22. Isla MI., Moreno MI., Sampietro AR., and Vattuone MA.. Antioxidant activity of Argentine propolis extracts. Journal of Ethnopharmacology, 2001; 76: 165-170.
23. Medic-Saric M., Rastija V., Bojić1 M., and Maleš Z. From functional food to medicinal product: systematic approach in analysis of polyphenolics from propolis and wine. Journal of Nutrition, 2009; 8: 33.
24. Simões LM.,Gregório LE., de Souza ML., *et al*. Effect of Brazilian green propolis on the production of reactive oxygen species by stimulated neutrophilis. Journal of Enthropharmacology, 2004; 94: 1: 59-65.
25. Kujumgiev A., Tsvetkova I, Serkedjieva Y, Bankova., *et al*. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology, 1999; 64: 235-240.
26. Marcucci MC. Propolis: chemical composition, biological properties and therapeutics activity. Apidologie,1995; 26: 83-99.
27. Kharidia R., and Liang JF. The activity of a small lytic peptide PTP-7 on Staphylococcus aureus biofilms. Journal of Microbiology, 2011; 49; 4; 663-668.
28. Kumasawa S., Hamasake T., and Nakayama T. Antioxidant activity of propolis of various geographic origins. Food Chemistry, 2004; 84: 329-339.