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Evaluation of the Antimicrobial Activity of Propolis Ointment Against *Staphylococcus aureus*

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Abstract

Background: This study was carried out to determine the antimicrobial effect of propolis ointment formulations against *Staphylococcus aureus.*

Methods: Propolis was extracted with 70 ml Ethanol (99 %) and the ointment was prepared by incorporating the extract into emulsifying ointment BP. Formulations were evaluated against *Staphylococcus aureus* using agar diffusion test. Physicochemical properties of the formulation (pH, viscosity and spreadability) were determined using a pH meter, and viscometer. Homogeneity and texture were also determined. Differences in means were evaluated by the independent student t-test at p-value < 0.05.

Results: The ointment was dark brown, greasy with a smooth texture. The viscosity of the propolis emulsifying ointment and simple ointment was 11133 ± 1229 and 11800 ± 1100 cP with spreadability of 6.32 ± 1.33 and 6.00 ± 2.40 mm^2/g respectively. The pH of the simple and emulsifying ointment was 7.10 ± 0.05 and 7.22 ± 0.06 respectively. There was however reduced pH, spreadability and viscosity of the formulation over a period of 90 days (3 months) resulting in more acidic preparations less than 6 (5.65) in batch F_2. Depth of penetration of the simple ointment was between 6.00 ± 0.8 mm to 7.55 ± 0.8 mm and 6.75 ± 0.5 mm to 9.20 ± 1.10 mm for the emulsifying ointment over a period of 14 days. The minimum inhibitory concentration of the propolis extract against Staphylococcus aureus was 300 mg/ml. Inhibition zone diameter of the simple and emulsifying ointment were 16.5 ± 1.3 mm and 17.0 ± 1.2 mm respectively.

Conclusion: Propolis can be formulated as an ointment for the treatment of bacterial skin infections. The physicochemical properties of the formulation were stable with gradual decline over a period of 3 months. The propolis ointment exhibited antimicrobial activity against *Staphylococcus aureus*.

Keywords: Propolis ointment, Antibacterial activity, Ointment properties

INTRODUCTION

Propolis is a hydrophobic, hard and brittle material produced by bees. With heat, it is soft, pliable, gummy, and very sticky (Hausen *et al.,* 1987). It has a distinct aromatic smell with variability in colour depending on the source and age (Bankova *et al.*, 2000). Propolis of different origin contains different constituents. Propolis is obtained from plant-derived compounds by bees and this contributes to its complexity and variability (Vigay, 2013). Crude propolis is made up of 50 % resins, 10 % essential oils, 30 % waxes, 5 % pollen and 5 % of other compounds (Park *et al.,* 2002; Pietta *et al.,* 2002). Propolis is derived from different plant parts, such as buds, flowering buds, and resinous exudates, which are collected from bees and taken for use in the beehive (Sforcin, 2016). The activity of these composites depends on a plethora of factors. The different solvents employed in the extraction processes dictate the activity of the biologically active constituents in propolis. This is as a result of the differences in solvent polarity and it is in part responsible for its diverse pharmacological activity (Ugur and Arslan, 2004). The principal compounds implicated in its biological activities include polyphenols, aromatic acids, and diterpenic acids, but very few different propolis types have been different in their main bioactive compound (Vijay, 2013). Propolis has been found to have anti-bacterial, anti-inflammatory, anti-viral, anti-oxidant, anti-protozoan, anti-tumor, anti-fungal, anti-septic, anti-mutagenic, anti-hepatotoxic activity in addition to cytotoxic activity (Sforcin, 2016; Toreti *et al.,* 2013).

Pertinent to note is that propolis produced in the northern hemisphere results from bee activity during spring, summer, and the beginning of autumn, and this translates to seasonal differences in chemical constituents. As a result of favourable climate in the southern hemisphere, propolis is produced by bees almost throughout the year. Calegari *et al*., (2017) reported differences in propolis collected in the southern state of Parana, Brazil, from March–June 2013 and in March 2015. The stronger antioxidant activity observed with the former was due to the large amount of phenolic compounds present. Additionally, due to the enormous biodiversity of propolis, the varied composition makes it difficult to pinpoint the origin of a particular propolis. Recent research had classified propolis based on its geographical location of origin such as Brazilian, Greek, and Indian propolis (Wang *et al*., 2016).

However, classifications based on colour had been done (such as green, brown, and red propolis), as well as agricultural characteristics ("organic" propolis) (Zhang *et al*., 2016). Zhang *et al*., (2016) have also classified propolis based on region/flora. We have seven types of propolis, according to the plant sources, including poplar propolis (Populus spp.), Eucalyptus propolis (AbuMellal *et al*., 2012), Baccharis propolis (Dos Santos Pereira *et al*., 2003), Brazilian green propolis, Clusia (Hernandez *et al*., 2005) (Brazilian red propolis), Macaranga propolis (Huang *et al*., 2007) (Taiwanese green propolis), Betula propolis, and Mediterranean propolis (Popova *et al*., 2010). The botanical source of Nigerian propolis has been identified by Omar *et al.,* (2016, and 2017) to belong to Dalbergia and Macaranga spp. There is a scarcity of scientific study on propolis. Propolis is gaining world-wide recognition in contemporary medicine, veterinary medicine, pharmacology, and cosmetics, where it may be manufactured as capsules, lozenges, creams, gels, emulsions, and ointments due to its wide range of capabilities. As a result, the goal of this research is to formulate a propolis ointment and test its antibacterial effectiveness against gram-positive bacteria (*Staphylococcus aureus*). Staphylococcus aureus (SA) was chosen because of its dominance on the skin as the most common host.

METHODOLOGY

Materials

Propolis was harvested from the Faculty of Agriculture, University of Benin, Benin city, Edo state. Ethanol (99.9%), white soft paraffin (Unicorn Petroleum Industries Pvt, Ltd, India), liquid paraffin (Unicorn Petroleum Industries Pvt, Ltd, India), emulsifying wax (Unicorn Petroleum Industries Pvt, Ltd, India), polysorbate 80 (Merck Pharmaceuticals, USA), wool fat (SigmaAldrich, USA), cetosteryl alcohol (Prakash Chemicals International Pvt. Ltd) Cod liver oil (SigmaAldrich, USA) acacia powder (Loba Chemie Pvt. Ltd, Mumbai, India), isolate of *Staphylococcus aureus* was obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City; viscometer (Brookfield Technologies, UK) pH meter, pipettes, glass plates and all used chemicals were within analytical grade limits.

Extraction of propolis

The propolis was collected and ground into granules. About 50 g of the chopped propolis was soaked in 150 ml of 99.9 % ethanol by maceration over a two

week in a 500 ml glass jar and kept in a dark room with consistent shaking. The extract was thereafter filtered using a glass funnel to obtain a dark brown filtrate which was later concentrated.

Preparation of simple ointment base

Ointment bases were prepared by fusion method as described by Alalor *et al.,* (2012). Hard paraffin (1.5 g), wool fat (1.5 g), cetostearyl alcohol (1.5 g), white soft paraffin (25.5 g) were weighed into a porcelain dish and melted over a hot water bath in decreasing order of their melting points to produce 30 g simple ointment. On removal, 300 mg of propolis was incorporated into the 30 g simple ointment base and stirred continuously obtain a brown paste like mass and labeled F_1 (Table 1)

Preparation of emulsifying ointment base

Emulsifying wax (9 g), white soft paraffin (15 g) was weighed into a porcelain dish and melted over a water bath. Thereafter, 6.98 ml of liquid paraffin was added and stirred continuously to produce 30 g emulsifying ointment base. Propolis (300 mg) was incorporated into the emulsifying ointment base and was stirred continuously over a cold bath to a brown paste which was labeled as F_2 (Table 1)

Table 1: Formulation table

Ingredients	Simple emulsifying ointment 30 g BP (F_1)	Emulsifying ointment BP 30 g (F_2)
Hard paraffin	1.5 g	-
Wool fat	1.5 g	-
Cetostearyl alcohol	1.5 g	-
White soft paraffin	25.5 g	-
Propolis extract	300 mg	300 mg
Emulsifying wax	-	9g
White soft paraffin	-	15 g
Liquid paraffin	-	6g (6.98 ml)
Total	30.3 g	30.3 g

Physical Properties of the Ointment

Physical properties which include appearance, texture and greasiness were assessed (Gehan *et al.,* 2014).

Spreadability

Propolis ointment formulations (1 g) was weighed and placed between two glass plates of the same dimension (10 cm x 20 cm). Twenty-five grams (25 g) of weight was put onto the top plate and left there for one minute. Determinations were done in triplicates and the spreadability factor (S_f) was calculated using the formula below

$S_f = A/W$ …….Equation 1

Where, S_f is spreadability factor, A is total area (mm^2) and W is total weight (g).

Determination of pH

The pH was determined using the method of Gehan *et al.,* (2014) with modifications due to the viscosity of the ointment. Five (5 g) grams of ointment formulation were diluted to 10 ml with de-ionized water in a volumetric flask. The pH was recorded using a pH meter. Determinations were carried out in triplicates and the mean result recorded

Viscosity Measurement

The rheological behaviour of the formulations was evaluated by viscosity measurement. The viscosity in centipoise (cP) was determined by CAP-2000 Brookfield viscometer using the modified method of Akanksha *et al.,* (2009). Test sample was weighed in a clean and dry 250 ml beaker, and the viscosity of the test sample was determined using spindle 5 following standard operating procedures at 50 rpm. Samples were measured at 27 ± 1°C.

Depth of Penetration of the formulation

A plunger of length 15.0 cm, weight 0.6 g and thickness 1.5 cm was allowed to fall freely from a fixed height of 42.0 cm into the sample holder containing ointment. This was left for 10 sec after which the plunger was removed and the depth of penetration was measured with the aid of a pair of

divider. Determinations were carried out in triplicates and average taken.

Stability of the Ointment Formulation

The stability of the formulation was assessed by evaluating the pH, viscosity and spreadability over a period of twelve (12) weeks. The pH, viscosity, and spreadability was measured at the first day which was designated as day 0, followed by day 7, 14, 30, 60 and 90 at room temperature. Room temperature was chosen because of the cost implications of providing special cold storage conditions in Nigeria.

Determination of minimum inhibitory concentration (MIC) of propolis extract

The minimum inhibitory concentration of the extracted propolis was determined using the cup plate method. Different concentrations of the propolis extract which includes 100, 200, 300, 400, 500 and 1000 mg/ml were prepared by dissolving in water and polysorbate 80. The nutrient agar was poured into a petri dish and allowed to set. After setting, a loop full of the organisms from the Mcfarland standard was streaked across the plate. Thereafter, a 10 mm cork borer size was used to bore six (6) holes equidistantly into the already inoculated plate and labeled appropriately. The wells were sealed with molten agar to cover any cracks. The different concentrations of the propolis were placed into the wells. The plates were then incubated at 37 ºC for 24 h in an incubator after which the inhibition zone diameter was measured and minimum inhibitory concentration determined.

Evaluation of the antibacterial activity of propolis ointments

Freshly prepared sabouraud agar was poured into four different petri dishes. Each petri dish was labeled SA. After setting, each plate was streaked with *Staphylococcus aureus*. Four wells of 10 mm in diameter were bored equidistantly on the plate using a sterile cork borer. The wells were completely filled with 1 g of the ointment using a plunger and a blank ointment base (without propolis). The cultures were incubated at 37 ºC for 24 h and the inhibition zone diameter was measured.

RESULTS

Physical Properties of the Ointment

Variations in seasonal, hive, geographical and plant source of propolis influence its physical properties (Ahmed *et al.,* 2017). The botanical source of Nigerian propolis has been identified by Omar *et al.,* (2016, 2017) to belong to Dalbergia and Macaranga spp. The colour varies from yellow to dark brown. However, it is also dark in colour depending on the sources of resin found in the particular hive area. The Blank preparation was white due to the absence of propolis in the formulation, smooth and greasy. The propolis ointment was dark brown in color, smooth to touch, greasy and with a characteristic odor (Table 2).

Table 2: Physical properties of propolis ointment

Formulation identity	Physical appearance	Texture	Greasiness
Propolis simple ointment (F_1)	Dark brown	Smooth	Greasy
Propolis emulsifying ointment (F_2)	Dark brown	Smooth	Greasy
Simple ointment BP (Blank 1)	White	Smooth	Greasy
Emulsifying ointment BP (Blank 2)	White	Smooth	Greasy

As noted in previous studies, the characteristics taste and odour of propolis has been attributed to Artepillic C (Taketoshi *et al.,* 2012). The dark brown color of the propolis extract was imparted on the ointment due to the color of the propolis itself.

Physicochemical properties of propolis ointment formulation

The pH, spreadability and viscosity of the ointment are presented in Table 3. The ointments had higher pH than the blank preparations. The appearances and presence of coarse particles were used to evaluate the texture and homogeneity of the formulations. The homogenous and appealing appearance indicated that there were no signs of phase separation, physical or chemical instability. The spreadability of semisolid formulations is the ability of the preparation to evenly spread on the skin, and it is an important

aspect to consider in administering topical preparations. The spreadability values, that's the diameters observed for the formulations, after five minutes is an indicator of spreadability characteristic. The values refer to the extent to which the formulations readily spread on the application surface by applying a small amount of shear (Vijay *et al.,* 2013). The blank preparations had a higher spreadability than the propolis formulations. Propolis tends to behave like glue thus reducing the spreadability of the formulation. The emulsifying ointment formulation had a higher spreadability than the simple ointment formulation, however the differences were not statistically significant (p-value 0.318). The viscosity of the simple ointment was higher than the emulsifying ointment and the difference was statistically significant (p-value 0.001). This is due to the presence of liquid paraffin which is thought to lower viscosity (Ewa *et al.*, 2014). The pH of the formulations slightly increased when the active ingredients were added to the bases. The pH of the skin is between 4 to 6. The pH range for topical preparations should be between 6.8-7.5 at 25 ^{o}C and this depends on type of the formulation used. Increase in pH increases dehydrative effect, irritability and proprioni bacteria count (Baranda *et al.,* 2002). The pH of the ointments was within the pH of the skin, which indicated that it is suitable for application on the human skin with no risk of skin irritation or burns. The differences in pH, spreadability factor of the formulation was not statistically significant.

Table 3: Physicochemical properties of propolis ointment formulation

Formulation	pH	Viscosity (cP)	Spreadability factor (mm^2/g)
F_1	7.10 ± 0.05	11800 ± 1100	6.00 ± 2.40
F_2	7.22 ± 0.06	11133 ± 1229	6.32 ± 1.33
Blank 1	6.73 ± 0.05	15260 ± 1645	7.25 ± 3.85
Blank 2	6.90 ± 0.03	13430 ± 1320	6.60 ± 2.66
p-value	0.324	0.001*	0.318

Significance: p-value < 0.05. F_1 = Propolis simple ointment, F_2 = Propolis emulsifying ointment, Blank 1 = Blank simple ointment, Blank 2 = Blank emulsifying ointment

Depth of Penetration of Propolis Ointment

Changes in depth of penetration of the preparation during storage at room temperature are given in Table 4. There is reduced penetration of the plunger with time. The differences in penetration of the plunger into the various formulations are as a result of their varying viscosities and this was found to be statistically significant. The plunger moved slower in the simple ointment formulation as against the emulsifying ointment because of the greater viscosity of the former. There were significant differences in the depth of penetration over a period of 2 weeks (p-value < 0.05).

Table 4: Depth of penetration (mm) of Propolis Ointment

Formulation	Day 1	Day 3	Day 9	Day 14	p-value
F1	7.55 ± 0.80	6.95 ±1.23	6.48 ± 0.50	6.00 ± 0.80	<0.001*
F2	9.20 ± 1.10	8.68 ± 2.00	7.00 ± 2.30	6.75 ± 0.50	0.001*
Blank 1	10.80 ±1.10	10.44 ± 2.00	9.36 ± 1.50	8.80 ± 2.00	<0.001*
Blank 2	10.20±1.00	9.40 ± 1.80	8.12 ± 2.00	7.44 ± 1.50	0.001*

**Significance: p-value < 0.05. F_1 = Propolis simple ointment, F_2 = Propolis emulsifying ointment, Blank 1 = Blank simple ointment, Blank 2 = Blank emulsifying ointment*

Stability Testing

The stability of the formulation was evaluated using the pH, viscosity and spreadability over a period of three months at room temperature is shown in Figure 1a to 1c below: The pH, spreadability values of the formulations did not change significantly over the period of 12 weeks indicating chemical stability. However, there was significant reduction in viscosity over the same period.

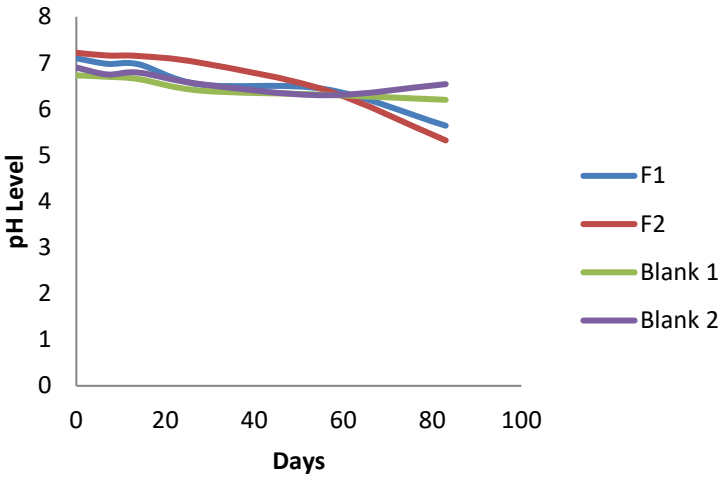

Fig 1a: pH of the formulations

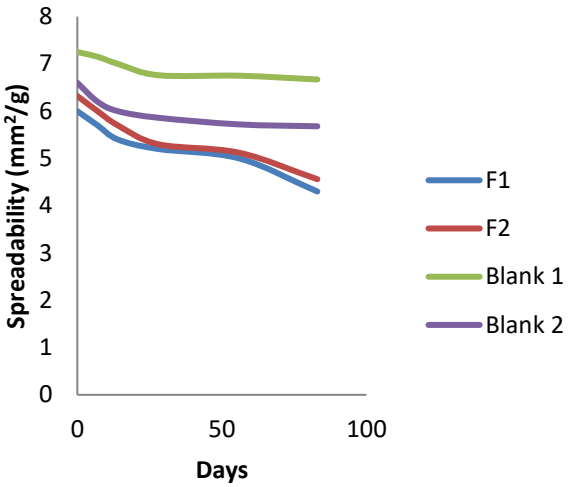

Fig. 1b: Spreadability of the formulations

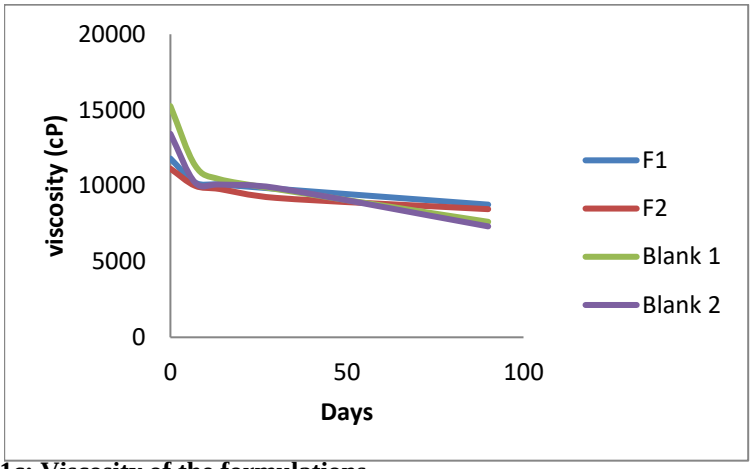

Fig. 1c: Viscosity of the formulations

Preliminary screening of the antimicrobial activity

The preliminary antibiotic screening of propolis ethanol extract against *Staphylococcus aureus* is given in Table 5. The Minimum Inhibitory Concentration (MIC) is the minimum concentration of a sample that can inhibit the growth of an organism. Antimicrobial inhibition of the extract was concentration dependent with higher concentrations of propolis giving higher values of inhibition zone diameter. Studies had shown that propolis inhibited gram positive bacteria more than gram negative bacteria (Izabela and Tomasz, 2019). Serial dilutions of the sample were used to determine the MIC that is the lowest concentration of material that would still show antibacterial properties. From the result shown in Table 4, 100 mg/ ml concentration showed no inhibition. Inhibition zone diameter (IZD) was obtained in 200, 300, 400, 500 and 1000 mg/ml concentration of the extract. The inhibition produced by the 200 mg/ml concentration was not satisfactory as growth was still observed. The 300 mg/ml concentration of extract produced appreciable IZD with no growth. The 400, 500 and 1000 mg/ml concentrations showed even greater inhibition as shown in the results, however, the 300 mg/ml concentration of extract was chosen as minimum inhibitory concentration because it was the lowest concentration that showed satisfactory inhibition zone diameter against *S. aureus.* This MIC was far higher than that of Popova *et al.*, (2017) using dichloromethane extract (MIC values of 0.90–1.34 mg/ml). Thus, the solvent used in the extraction of phyto-constituents from propolis (due to varying poliarity), the extraction time, time of collection, variability across geographical locations are factors

that influence its *in vitro* activity. The inhibition zone diameter produced at different concentrations was statistical different. This had shown that the inhibition was concentration dependent.

Table 5: Inhibition zone diameter produced by the crude propolis extract

Concentration (mg/ml)	Inhibition zone diameter (mm)
100	Nil
200	5.2 ± 1.1
300	17.3 ± 1.3
400	18.4 ± 1.2
500	19.2 ± 1.2
1000	20.4 ± 1.3
p-value	0.004*

**Significance: p-value < 0.05*

In Table 5 above, the minimum inhibitory concentration of the propolis extract against *Staphylococcus aureus* was 300 mg/ml. At 200 mg/ml, the inhibition was not satisfactory and the difference in the mean of the inhibition zone diameter of the extract was statistically significant.

Previous studies of Kubina *et al.,* (2015) on Polish propolis gave an MIC in a range of 0.39–6.25 mg/ml when tested against *Staphylococcus epidermidis*. Another study on an ethanolic extract of Polish propolis displayed varying effectiveness against 12 methicillin-sensitive and -resistant *Staphylococcus aureus* with a MIC within the range of 0.39 to 0.78 mg/ml (Wojtyczka *et al*., 2013). The differences in MIC can be attributed to the variability in the constituents of propolis across countries as well as the time of collection as posited by Calegari *et al*., (2017). In addition, the solvent of extraction and micro-organisms used in the study would also determine the MIC.

Antimicrobial screening of propolis ointment against *Staphylococcus aureus*

The zone of inhibition of the emulsifying ointment was higher than that of the simple ointment (Table 6). No inhibition was observed for either blank preparation due to the absence of propolis in the formulations. The inhibition produced by the propolis emulsifying ointment was 0.5 mm greater than that of the propolis simple ointment. However, the inhibition zone diameter of the simple and emulsifying ointment were similar to that of the extract indicating that the ointments bases did not alter the antimicrobial activity of the extract hence their suitability for formulation.

Table 6: Inhibition zone diameter of propolis ointment formulations

Ointment formulation	Inhibition zone diameter (mm)	p-value
F_1	16.5 ± 1.3	0.015
F_2	17.0 ± 1.2	
Blank 1	NIL	
Blank 2	NIL	

Significance: p-value < 0.05

The antibacterial activity of propolis is a combined effect of protein synthesis inhibition and bacterial growth by inhibiting cell division (Bankova, 2005; Wojtyczka *et al*., (2013). The activity is attributed mainly to the high content of flavonoids such as galangin, pinocembrin and pinobanksin, which have been studied to possess high antimicrobial (antibacterial as well as fungicidal) activity (Popova *et al*, 2017). Galangin and caffeic acids inhibit bacterial growth and cell division. Some active substances can also cause partial bacteriolysis as a result of cytoplasmic and cell wall distortion. Flavonoids had been extensively studied to affect bacterial membrane potential and cause permeability alteration within the inner microorganism membrane (Wojtyczka *et al*., 2013). However, for gram-positive

organisms, the presence of α-Mangostin inhibited *S. aureus* growth by disrupting the cytoplasmic membrane and preventing biofilm formation (Koh *et al.*, 2013). The propolis extract simple ointment and emulsifying ointment exhibited antibacterial activity against *Staphylococcus aureus.*

CONCLUSION

The simple and emulsifying ointment formulations were stable with good spreadability, pH and viscosity. Over a period of 90 days, there was decline in pH, viscosity and spreadability with better profile observed within the first month. The propolis ointments were successful in exhibiting activity against *Staphylococcus aureus*.

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