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Preparation and evaluation of a propolis ointment for skin nourishing and healing

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Abstract: Propolis is a resinous substance containing aromatic acids, esters, volatile compounds, hydrocarbons, steroids, enzymes, flavonoids, vitamins and minerals. In this direction it has nutrient, antibacterial and antioxidant properties due to its complex structure and content. In this study it was aimed to prepare a propolis ointment with a base of occlusive and emollient properties for nourishing and healing of skin damaged by environmental effects. Six formulations were prepared by using vaseline, lanolin, glycerine and different types of crude propolis. According to physical examinations only the ointment formulations prepared with ethanolic propolis extracts at two different concentrations were found as successful. Successful formulation including 1:5 propolis: Ethanol extract was evaluated by physical controls, mechanic tests, antimicrobial tests and stability evaluation. Results showed that prepared propolis ointment has a creamy yellow colour with an acceptable odour, homogeny and a good spreadability texture and has a good stability at 40°C and 25°C and for 2 and 3 months respectively. And it presented mild antibacterial effects on *E. coli* and *S. aureus* strains, which was acceptable for a frequent use for cosmetic purposes. As a conclusion prepared propolis ointment can be a good candidate for a cosmetic ointment for skin nutrition and healing purposes.

Keywords: Propolis ointment, nourishing, healing, texture analysis, stability.

INTRODUCTION

Propolis is a resinous substance that can be in different colours and is obtained by honey bees (*Apis mellifera*) from fissures in the leaves, flower buds, stems, and wood of many different tree species. The bees bring these ingredients back to the hive where they combine them with beeswax to create a very sticky substance (Pobiega *et al*., 2019). The content of propolis varies depending on the geography where it is produced, the type of bee, the mode of production and the fauna of the region. However, in general, all propolis contains compounds with high antimicrobial and antioxidant effects. In a study, it was stated that more than 420 kinds of chemical contents were found in propolis obtained from different geographies (López *et al*., 2014). The main ingredients of propolis are aromatic acids (for example, cinnamic acid, caffeic acid, ferulic acid), aromatic esters (cinnamic and caffeic acid ether esters), volatile compounds (geraniol, nerol, farnesol, β-eudesmol), aromatic compounds (vanillin), hydrocarbons (eicosan, tri-cosan, pentacosan), steroids (cholinesterol, fucosterol, stigmas-terol), enzymes (α-amylase, β-amylase), flavonoids (tecto-chrysin, pinobanksin, pinocembrin, chrysin, galangin, apigenin, kaempferol), acids (palmitic acid, melissic acid, cerotic acids), micro and macro nutrients (Ca, K, Mg, Na, Zn, Fe, Mn, Al, Ba, Cl), vitamins (B1, B2, B6, C, E) and volatile oil compounds (Kubiliene *et al*., 2014). In recent years, studies and research on propolis have increased considerably in terms of internal food supplement and external medical purposes (Namratha *et al*., 2013; Afkhamizadeh *et al*., 2018; Rojczyk *et al*., 2020; Staniczek *et al*. 2021; Furukawa *et al*. 2021; da Rosa *et al*. 2022). There are also some propolis combined with beewax and some other herbal substances such as almond oil, olive oil, sunflower oil, etc. including ointments on the market with treatment or skin care claims. Between those a medicine licenced propolis ointment has not been observed, and the cosmetic ones have different originates propolis combined with some herbal substances. Since obtained organic propolis from Sivas region has a very rich content approved with a licence, we aimed to prepare a different formulation other than the ones on the market. Considering the antibacterial and antioxidant properties of propolis, it has been predicted that topical ointment formulations for external use can be prepared for skin care. It was thought that it would be appropriate to use occlusive vaseline and lanolin, as well as humectant and emollient glycerine, which are suitable base components for both skin care and topical application in the formulation of ointments (Villiers *et al*. 2009) containing propolis. Due to the complex, resin and waxy structure of raw propolis, it is planned to add different processed types of raw propolis to the ointment bases to be prepared in the formulation design. Accordingly, in this study, first-step

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formulations containing an ointment base consisting of vaseline-lanolin-glycerin combination and ethanolic extract of crude propolis (Kayabaşı *et al*., 2019 and Yıldız *et al*. 2020), lyophilized form and unprocessed waxy form were prepared and organoleptic. After first step organoleptic controls propolis ethanolic extract included ointment formulations was found to be suitable for further evaluations and concentrated propolis ointment was evaluated by physical controls, texture tests, antimicrobial examination and its stability (Mandeep *et al*. 2011, Dhyani *et al*. 2019, Sagiroğlu, *et al*. 2020).

MATERIALS AND METHODS

Materials

Organic certificated crude propolis purchased from Engür A.Ş. (Sivas, Türkiye), ethyl alcohol, vaseline, lanolin and glycerine (Merck, Germany) were all pharmaceutical grade. Lethheen Broth AOAC (BiolifeItaliana Microbiology, Italy), Cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories), RPMI-1640 medium (Sigma) buffered to pH 7.0 with morpholine propane sulfonic acid (MOPS, Sigma), and tryptic soy agar (TSA, Difco Laboratories) mediums were obtained for the microbiological tests.

Methods

Processing of Raw Propolis

In this study, in order to facilitate grinding due to the extremely sticky nature of propolis, propolis samples were frozen at -18℃ for 24 hours, and the frozen propolis for grinding was ground in a laboratory ball mill (MSE Technology, Türkiye) until it turned into powder. For wax removal, the ground raw propolis was transferred to trays and kept in an oven at 60℃ for 120 minutes. The wax, which solidified again with the effect of the cold, was separated from the propolis by mechanical treatment. For the preparation of alcoholic propolis extract, the same procedure was followed for dewaxed and unwaxed propolis, and the method used in the studies of Kayabaşı (Kayabaşı *et al*., 2019) and Yıldız (Yıldız *et al*. 2020) was modified and applied. For this purpose, 100 g of ground propolis was subjected to extraction and periodic shaking at 50℃ for 5 days in 1000 ml (70 w/w) ethyl alcohol (145 rpm/15 min per hour). At the end of the 5th day, the extract was first subjected to coarse filtration (nonwoven fabric 50 µ) and then filtered through filter paper with a pore diameter of 20 µ to obtain ethanolic propolis extract. Propolis dense extract was obtained by condensing the extract prepared with 1:10 ethanol: propolis ratio by evaporating the alcohol to 1:5 ratio with the help of evaporator.

Preparation of Ointment Formulations Including Propolis

For propolis ointment formulations, 10% propolis [waxed, unwaxed and ethanolic (1:10, 1:5)], 50% petrolatum, 30% lanolin and 10% glycerine were used and six formulations were prepared. Cold and hot preparation methods were used as the ointment preparation method (table 1).

For F1 and F2 formulations; F1 and F2 were prepared by mixing glycerine and propolis at the rates specified in the formulation in glass mortar, adding lanolin and vaseline to it. For F3 and F4 formulations; F3 and F4 were prepared by adding lanolin and vaseline on a water bath in which glycerine and propolis were mixed at the rates specified in the formulation in porcelain capsules, and after the mixture melted at 50℃, by mixing. For F5 and F6 formulations; F5 and F6 were prepared by adding glycerine and vaseline after mixing alcoholic propolis with lanolin in glass mortar at the rates specified in the formulation and mixing.

Texture analysis of propolis ointment

Texture profile analyses of the ointments prepared with ethanolic propolis extracts were performed using a TA-XT Plus texture analyser (Stable Micro Systems, Surrey, UK) in compression mode. Back extrusion method was applied to determine the mechanical parameters such as firmness, consistency, cohesiveness and work of cohesion using back extrusion cell (A/BE) with 35mm disc and extension bar with 5 kg load cell. Before beginning the test, the instrument was calibrated with a 2-kilogram weight. The ointments were stored at 25°C±0.5°C and then filled into a 50 mm diameter back extrusion container to about 75% capacity. Centrally over the sample container, the extrusion disc was placed. The probe was calibrated to a distance of 10 mm, above the sample surface. The container is held to keep it from lifting as the probe takes its route back to the start. The instrument was set to the parameters mentioned in table 2.

Spreadability is the deformation under an external load, and it is a more dynamic quality. Compression test measures sample hardness by the force necessary to generate a certain deformation under a particular force. Spreadibility method was applied to determine the work of shear parameter using TTC spreadability rig (HDP/SR) with 5/25kg load cell and a heavy-duty platform (HDP/90). The ointments were stored at 25°C±0.5°C and then placed into the female cone which is found on the heavy-duty platform and pressed down to eliminate air pockets. After male cone probe calibration, sample holder was filled and further sample was removed with a knife in order to create a flat test surface. The instrument was set to the parameters mentioned in table 3. The experiments were done triplicate. Data collection and calculation were performed using Exponent Connect Software, Version 8.0.8.0.

Microbiological Evaluation Studies

Microorganisms: The American Type Culture Collection (ATCC) standard strains of *Staphylococcus aureus* ATCC

29213, *S. epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153 and as a representative of fungi, the yeast, *Candida albicans* ATCC 10231 were used in the studies. Bacteria and C. albicans inoculums were made as 0.5 McFarland overnight cultures, yielding concentrations of 1 x 10^8 and 1 x 10^7 colony-forming units (cfu/ml), respectively.

Agar Diffusion Assay: To determine the in vitro antimicrobial activities of the products and propolis, agar diffusion method was used as a pre-screening test against above mentioned microorganisms. Briefly, using the spread plate method, 100 µl of microorganism suspensions (equal to 0.5 McFarland) were inoculated into sterile petri plates over plates containing solidified agar medium and allowed to dry. Each 50µl of the products and propolis were transferred to approximately 8 mm diameter wells, which opened with a sterile pipette tip on the agar surface under aseptic conditions. Levofloxacin and fluconazole were used as positive control for bacteria and yeast, respectively. After keeping the Petri dishes at room temperature for 2 hours, the plates were placed into an incubator at 37°C for bacteria and at 35°C for yeasts. At the conclusion of the incubation, distinct inhibition zones were measured in millimeters for their diameter (Bauer *et al*., 1966).

Determination of Minimum Inhibitory Concentrations (MIC): *In vitro* antibacterial activities of products and propolis against *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 4352, *P. mirabilis* ATCC 14153; and antifungal activities against *C. albicans* ATCC 10231, were studied. For this purpose, MICs of the compounds were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012, CLSI 2016, CLSI 2020). Serial two-fold dilutions of products and propolis were prepared in Letheen broth for bacteria, and RPMI-1640 medium for yeast. In the test tray, each well was inoculated with 50µL of broth culture, resulting in a final concentration of 5 × 10^5 cfu/mL for bacteria and 5 x 10^3 cfu/mL for yeast. To avoid evaporation, the plates were closed and sealed in plastic bags. Then, the plates were incubated for yeast at 35°C for 48 hours and 37°C for bacteria for 18 to 24 hours. The MIC was defined as the lowest concentrations of compounds producing complete inhibition of visible growth. Rezasurin was used to colour microorganism's growth pink to show its proliferation. The reference antibiotics for bacteria and yeast were levofloxacin and fluconazole, respectively.

Stability testing

Stability testing was carried out by keeping 30 g of the ointments at 40ºC±1ºC for 2 months and another 30 g at 25ºC±1ºC for 3 months. It was controlled for any visual appearance and phase separation at the end of duration periods.

STATISTICAL ANALYSIS

Statistical analyses were performed by using Exponent Connect Software Version 8.0.8.0.

RESULTS

Six ointment formulations prepared and examined organoleptically. When F1 and F2 formulations were examined at the end of the preparation process, it was observed that ointment forms with heterogenous appearance and poor applicability to the skin. When F3 and F4 formulations were examined at the end of the preparation process, it was observed that inhomogeneous propolis forms melted with hot but did not disperse in the melted ointment base despite mixing. When the F5 and F6 formulations were examined at the end of the preparation process, it was observed that ointment forms were homogeneous, had an acceptable colour, odour and consistency according to organoleptic examination. Photos of F5 and F6 ointments were presented below in fig. 1.

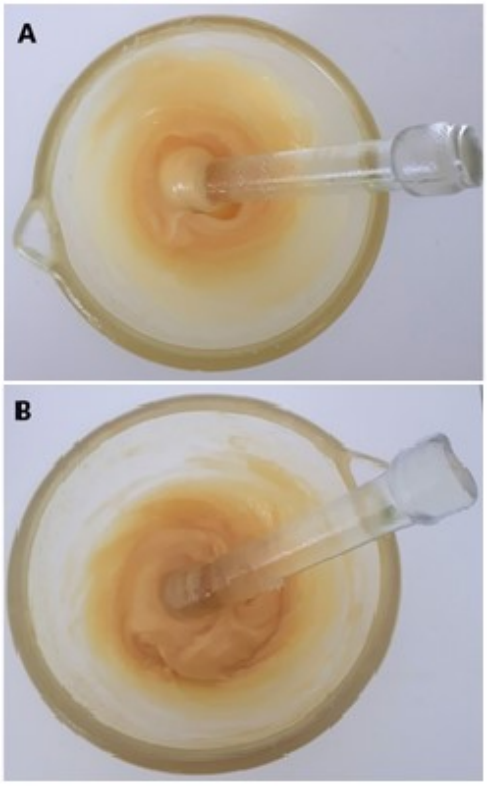

Fig. 1: Photos of A) F5 and B) F6 ointments.

The texture analysis results were given in table 4 and represented in fig. 2.

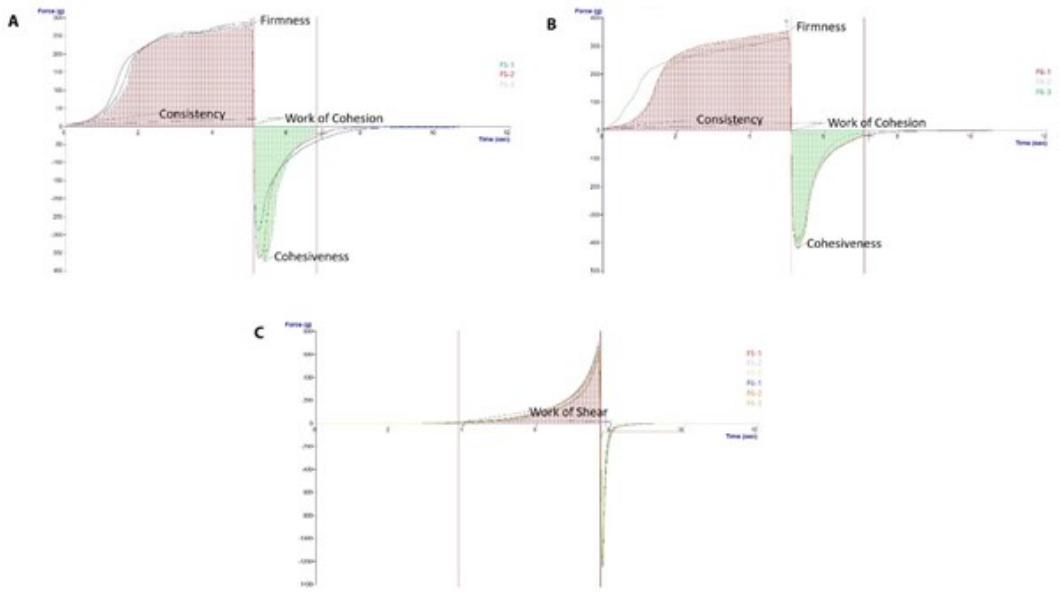

Fig. 2: Texture analysis results of A) F5 and B) F6 using back extrusion method and C) both F5 and F6 using spreadibility method.

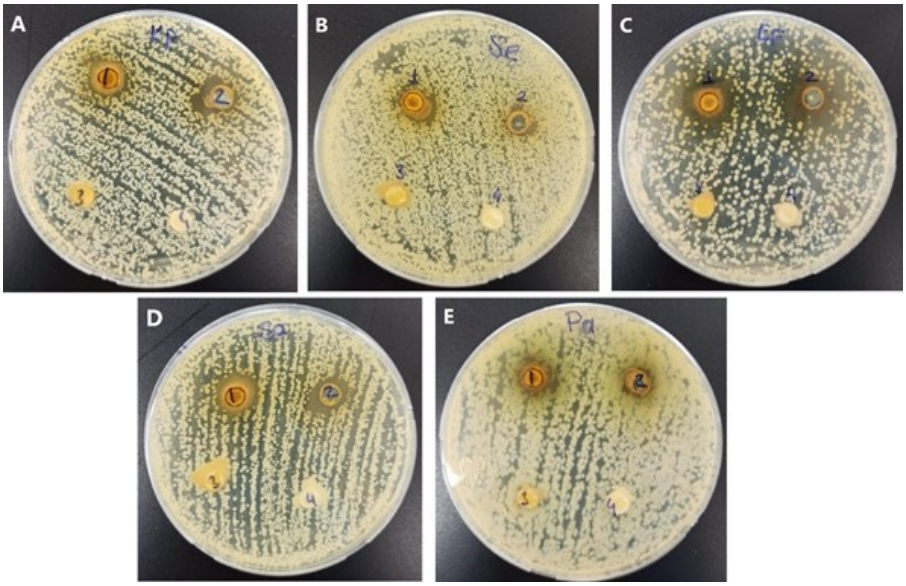

Fig. 3: Observed inhibition zones with 1) Propolis ethanolic extract (1:10), 2) Propolis ethanolic extract (1:5), 3) Propolis ointment formulation of F5 and 4) Propolis ointment formulation of F6 against A) *K. pneumoniae* ATCC 4352, B) *S. epidermidis* ATCC 12228, C) *E. coli* ATCC 25922, D) *S. aureus* ATCC 29213 and E) *P. aeruginosa* ATCC 27853 strains in plates.

Table 1: Ingredients and amounts of propolis ointments with codes and preparation methods

Ingredient/Formula Number	F1	F2	F3	F4	F5	F6
Propolis %10	With Wax	Without Wax	With Wax	Without Wax	Ethanol extract (1:10)	Ethanol extract (1:5)
Vaseline %50	√	√	√	√	√	√
Lanolin %30	√	√	√	√	√	√
Glycerine %10	√	√	√	√	√	√
Ointment Preparation Method	Cold mixing	Cold mixing	Hot melting (50℃)	Cold mixing (50℃)	Cold mixing	Cold mixing

Table 2: Parameters set on the texture analyser using back extrusion method.

Parameter	Set value
Test Mode	Compression
Option	Return to Start
Pre-Test Speed	2.00 mm/s
Test Speed	2.00 mm/s
Post-Test Speed	10.00 mm/s
Target Mode	Distance
Distance	10.00 mm
Trigger Type	Auto (Force)
Trigger Force	5.0 g
Break Mode	Off
Stop Plot At	Start Position
Tare Mode	Auto
Data Acquisition Rate	250 pps

Table 3: Parameters set on the texture analyser using spreadibility method.

Parameter	Set value
Test Mode	Compression
Test Speed	3.00 mm/s
Post-Test Speed	10.00 mm/s
Target Mode	Distance
Distance	23.00 mm
Trigger Type	Button
Break Mode	Off
Stop Plot At	Start Position
Tare Mode	Auto

Texture analysis results were shown in table 4. According to the results, the firmness, consistency and cohesiveness and work of cohesion values of F6 formulation were found to be higher than that of F5 formulation.

As a consequence of disk diffusion method for prescreening antimicrobial activity, it was seen that the propolis extracts had inhibition zones in diameters ranging from 10 mm to 18 mm against *E. coli*, *K. pneumoniae, S. aureus, S. epidermidis* and *P. aeruginosa*. Also, inhibition zones between 2-8 mm were formed with propolis ointment formulations (F5 and F6) against only *E. coli* and *S. aureus* strains. However, no inhibition zones formed against the other studied bacteria or yeast *C. albicans* with propolis extracts or ointments. Obtained inhibition zones were shown in fig. 3. As a result of microdilution assays of propolis extracts and the ointment formulations, there were no MIC values were detected.

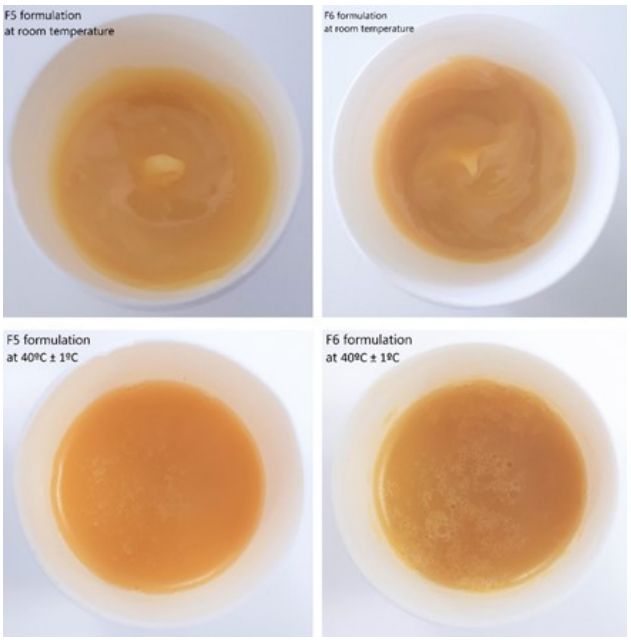

Fig. 4: Photos of F5 (a) and F6 (b) ointments after stability testing.

Results of stability testing that was performed for F5 and F6 propolis ointments was controlled for any visual appearance and phase separation at the end of duration periods. The photos of the ointments at the end of the stability testing were given in fig. 4.

DISCUSSION

Six ointment formulations examined organoleptically and according to the results F1 and F2 formulations showed heterogenous appearance and poor applicability to the skin were found to be unsuccessful. F3 and F4 formulations were inhomogeneous propolis forms melted with hot but did not disperse in the melted ointment base despite mixing. Therefore, ointment forms with poor applicability to the skin were obtained, and these formulations were also found to be unsuccessful. F5 and F6 formulations were homogeneous, had an acceptable colour, odour and consistency according to organoleptic examination, and therefore were highly applicable to the skin, were found to be successful. In line with the obtained results ethanolic propolis was accepted as compatible with ointment base.

Texture properties of semisolids are important parameters for pharmaceutical performance as well as for patient

Table 4: Texture analysis results of the ointments.

Samples	Firmness (g)	Consistency (g.s)	Cohesiveness (g)	Work of Cohesion (g.s)	Work of Shear (g.s)
F5	284.26 ± 3.77	927.66 ± 17.20	-341.53 ± 38.22	-245.61 ± 20.08	435.43 ± 7.69
F6	336.74 ± 10.54	1145.19 ± 45.64	-400.71 ± 13.69	-280.69 ± 14.29	543.83 ± 3.98

acceptance. Firmness relates to the viscosity of the product and defined as the maximum force in the plot of force versus time and the sample is more firmly defined the higher the value (Bhagurkar *et al*., 2016). The consistency is measured by the area of the curve up to this point; the higher the value, the thicker the consistency of the sample. Another indication of consistency or resistance to flow off the disc is provided by the negative region of the graph that is created on probe return because of the weight of the sample that is lifted mainly on the upper surface of the disc. The maximum force is used to determine the cohesiveness of the sample; the more negative the value, the more cohesive the sample is. The area of the negative area of the curve is known as the work of cohesion, and the higher the value the more resistant the sample is to withdrawal, which is an indication of the cohesiveness and consistency of the sample.

According to the results of the texture analysis of ointments, the higher firmness indicates that F6 formulation is more viscous and the higher cohesiveness, which is essential to keep the formulation adhered to application site, shows that F6 is more stuff than F5. The work of shear gives information about spreadability characteristics of the formulations obtained from texture analysis (Calixto *et al*., 2018). The semisolid formulation required less effort to spread over the surface, as indicated by the lower spreadability value, which indicates that only a modest amount of shear was needed to spread the formulation. Spreadability is crucial for patient compliance and ensuring that the formulations are applied uniformly to a broader area of skin (Yadav *et al*., 2014). The work of shear value of F5 formulation was found to be less than that of F6 formulation and thus F5 represented higher spreadability. Texture analysis results showed that both formulations possessed good firmness, cohesiveness, consistency and spreadability, which are necessary for application and retention on the skin to have optimum patient acceptance. The topical semisolids should be easily removed from the package but thick enough to maintain on the surface (Kaewbanjong *et al*., 2017). Hence, propolis extract ointments were found to possess all the attributes desired for topical application.

According to the microbiological evaluation studies it could be summarized as the propolis extracts and ointments exhibited slight antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The obtained antimicrobial activity results were conforming to those of Gezgin *et al*. (Gezgin *et al*., 2019) and Kuştarcı *et al*. (Kuştarcı et. al, 2011). Similarly, In the antimicrobial activity studies of propolis-containing topical skin preparations, it has been reported that there were limited effect against Gram positive bacteria *S. aureus*, and either no effect or a very low level of antimicrobial activity against yeast *C. albicans* (An et. al, 2021; Seibert *et al,,* 2019). It is thought that this situation is caused by the structural differences of the cell wall, which is the cell component that propolis acts on, both between Gram positive and Gram negative bacteria and between bacteria and yeast.

According to the stability testing results, at room temperature and 40ºC ± 1ºC, neither the visual appearance nor the phase separation changed. These results provide a preliminary idea of the compatibility and stability of the formulations.

CONCLUSION

Due to its complex structure and content, propolis has difficulties in terms of dispersion and mixing in various solvents and substrates. Among the different types of raw propolis, waxy and unwaxed ones were found to be unsuitable for formulation preparation and alcoholic extracts of propolis were found to be suitable for preparation of especially semisolid preparations. A propolis ointment has a creamy yellow colour with an acceptable odour, homogeny and a good spreadability texture, has a good stability with mild antibacterial effects on *E. coli* and *S. aureus* strains, which was acceptable for a frequent use for cosmetic purposes can be achieved. As a conclusion, prepared propolis ointment could be a good candidate in terms of nourishing and healing purposes for damaged skin caused by environmental effects such as weather-related damages (cold, wind or sun burn) or detergent damages.

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