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The Use of Natural Preservative Propolis and *Hypericum perforatum* Oil in Herbal Cream Production

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Abstract

Objective: Consumers' preference for products produced with natural additives has accelerated the search for natural substances that are alternative to synthetic materials in cosmetics and food production. The aim of the study is to obtain a herbal cream by using the natural preservative effect of propolis and the moisturizing effect of St. John's wort fixed oil of *Hypericum perforatum* plant which grows naturally in our country.

Material-Method: The chemical content of the obtained propolis ethanolic extract was determined by Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis. *Hypericum perforatum* fixed oil was obtained by maceration and the chemical content of the oil was determined by gas chromatography mass spectrometry (GC-MS) analysis. Herbal cream was prepared using PEG400 to facilitate dispersion of *Hypericum perforatum* oil and propolis extract obtained in water-based cream formulations and to provide a creamy consistency. Microbiological stability tests were carried out with reference to ISO21149, ISO16212, ISO18416, ISO22717 and ISO21150 standards and ISO11930: 2012 standard for challenge test (preservative efficacy tests).

Results: The total phenolic content of propolis ethanol extract was determined as 66.096 ± 1.546 mg gallic acid equivalent (GAE) / mL sample, dry weight and flavonoid content as 13.375 ± 0.185 quercetin equivalent (QE) / mL sample. An extract rich in phenolic components such as quercetin, p-coumaric acid, ferulic acid, trans-cinnamic acid, benzoic acid was obtained. Component analysis showed that propolis ethanolic extract could show preservative efficiency. The presence in the GC-MS analysis of *Hypericum perforatum* oil that it contains fatty acids widely used in the cosmetic and pharmaceutical industry clearly demonstrated that the oil can be used safely in cream formulations.

Conclusion: The test results of the creams were prepared using *Hypericum perforatum* and propolis clearly revealed that the obtained propolis extract provided protective efficacy. The thickener property of PEG400, which is used to ensure homogeneous distribution, reduced the need for an extra thickener additive.

Keywords: Natural Preservative, Herbal Cream, Propolis, *Hypericum perforatum* L., St. John's Wort

INTRODUCTION

Natural products are widely used in the treatment of diseases and in the food and cosmetic industries from past to present. With the discovery of the side effects of synthetic products and reporting the health hazards, the demand for natural products is increasing day by day. This has led to a rapid increase in search for natural products that can replace synthetic cosmetic additives. These natural products include widely used bee products and herbal products. In the present study, the natural preservative effect of propolis and the moisturizing and the wound healing effect of *Hypericum perforatum* oil were combined.

It is known that the therapeutic properties of

propolis have been used since ancient times. Like other bee products, propolis is widely used for the prevention and treatment of various diseases and is known to have rich biological activity. Propolis is also used as an alternative to synthetic preservatives in the cosmetics and food industry with its antibacterial, antifungal and antioxidant qualities [1]. The biological activities of the components in the propolis chemical composition have made propolis an important bee product. There are many studies showing that propolis has antimicrobial, antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, wound healing, tissue regenerating and anesthetic effects [2-5]. Studies on propolis have shown that this substance also contains vitamins, minerals and elements that are very important and essential for human health. Propolis also has anti-cancer, liver preservative, local anesthetic and antimutagenic activities [6,7]. Its antibacterial, antifungal and antioxidant qualities show that propolis can be an alternative additive to chemical preservatives [8].

Propolis contains 50% resin and herbal balm, 30% beeswax, 10% essential and aromatic oils, 5% pollen and 5% various organic components. Wax and organic components are usually removed by extraction. More than 300 ingredients have been identified in unprocessed propolis, including polyphenols, terpenoids, steroids, sugar and amino acids [9,10]. Propolis is beneficial for health since it contains high amounts of chemicals such as epicatechin, naringenin, catechin, genistein, kaempferol, chlorogenic acid, quercetin, apigenine, o-coumaric acid, protocatechuic acid, syringic acid, p-coumaric acid, gallic acid, ferulic acid, caffeic acid [5,10,11]. Hexane and ethanol extracts of propolis have been reported to show high antimicrobial activity against the tested microorganisms, as they contain the main phenolic components of pinbanxin and naringenin [12]. All these studies clearly reveal the suitability of propolis as a natural preservative.

Since propolis shows little solubility in water and hydrocarbon solvents and quite high in alcohols, its commercially available form is mostly 96% ethanol extract [7]. Usually it contains waxes, resins, water, inorganic ingredients, phenols and essential acids. The main biologically active ingredients of propolis are not very well soluble in water, oil and other solvents commonly used in the pharmaceutical industry. The active ingredients of propolis are easily soluble in ethanol, but ethanolic extracts cannot be used in the treatment of some diseases encountered in ophthalmology and pediatrics. Therefore, there are many studies investigating the chemical composition, radical scavenger and antimicrobial activities of propolis extracts, in which different solvents are used as extraction solvents. It is possible to obtain propolis extract with chemical content similar to ethanolic extract in extractions performed by adding additives that increase polarity such as vegetable glucose, sorbitol, glycerol and polyethylene glycol to water [13]. Comparing the total phenolic compound amount of polyethylene glycol 400 (PEG400) and water mixture or PEG400 extracts with olive oil-water mixture extract and ethanol extract, it was observed that the antimicrobial activity was equal or better than ethanolic extract. It was observed that the products of extractions performed in pure water or oil at room temperature did not show enough antimicrobial activity since they contain 5-10 times less amount of phenolic compounds [14]. In the study, PEG400 was used to increase the solubility of propolis in water-based cream formulations and to provide a homogeneous distribution. PEG400 is known to be a non-irritating hydrophilic substance that does not easily penetrate the skin. Since they are water soluble, they can be easily removed from the skin by washing with water. Aqueous PEG solutions can be used as suspending agents in topical ointments or to adjust the viscosity and consistency of other suspending agents [15]. For this reason, the use of PEG400 is suitable in the food and cosmetic industry to increase the solubility of propolis in water-based products.

Turkey is an agricultural country with a rich flora of medicinal and aromatic plants. *Hypericum perforatum* plant, which has been used in the treatment of many diseases since ancient times, is

a widely used plant to heal burns and wounds [16,17]. *Hypericum* species contain a large number of secondary metabolites, including naphrodiantrons, flurogonol derivatives, flavonoids, organic acids, essential oils, amino acids, xanthones, tannins, proxyanidines and other water-soluble components [18,19]. Although *Hypericum perforatum* has many bioactive compounds in its structure, the most active in terms of pharmacological properties are hyperforin, a phloroglucinol derivative, and hypericin, a naphthodianthron derivative [20]. Hypericin, a colored pigment that gives oil its red color, is the most important component [21]. It has been stated that oil prepared by maceration from *Hypericum perforatum* flowers reduces inflammation in wound healing, increases collagen synthesis in fibroblasts, positively affects epithelial regeneration, and has antibacterial and antiviral effects [16,22-25]. In studies where the extract obtained from *Hypericum perforatum* was applied topically and its effectiveness in wound healing was examined, it was found that the plant was quite effective in wound healing [26,27].

The use of *Hypericum perforatum* oil in wounds, burns, crushes and ulcers, pain relief and diuretic effects are supported by pharmacological research [28-32]. It has been reported that the ointment containing *Hypericum perforatum* oil shortens the burn healing time and shows antiseptic effect.

First-degree burns treated with the ointment healed within 48 hours, while second- and third-degree burns healed rapidly without scarring on the skin [26].

Propolis extract, which can replace synthetic substances used as preservatives in cream production, was used in the study. Propolis were obtained from beekeepers in three different regions in Duzce region of Turkey. The use of ethanolic extract was preferred because it is known that the active ingredients of propolis are easily soluble in ethanol. Since ethanolic extract was not dispersed homogeneously in water-based cream formulations, homogeneous distribution was achieved by using PEG400. PEG400 is known to be non-toxic. In addition to providing the distribution of oil-based additives in the water-based cream formulation, PEG400 also acted as a thickener in the formulation due to its thickener effect.

MATERIALS AND METHODS

Obtaining *Hypericum perforatum* oil (Maceration)

Three hundred grams of commercial olive oil was added over 100 grams of *Hypericum perforatum*. It was kept in a jar for 5 days with its lid open to see the sunlight and 45 days with its lid closed. In this process, the mixture was stirred in the jar every other day. After 50 days of maceration, the mixture was manually pressed through a cotton swab and dried over anhydrous sodium sulfate[33].

Hypericum perforatum oil content analysis

The maceration product was analyzed by GC/MS. The study was carried out on an Agilent 7890A GC System coupled to an Agilent 5975C inert MSD with Triple Axis Detector. Agilent HP5-MS (30 m × 0.25 mm × 0.25 µm) column was used as GC column. The oven temperature was held at 80 °C for 0 min., then ramped at 10 °C/min. to 130 °C for 1 min., then ramped at 10 °C/min. to 170 °C and held at this temperature for 0 min., then ramped 5 °C/min. to 215 °C for 12 min., then ramped 40 °C/min at 230 °C and held at this temperature for 3 min. The total run time was 31.87 min. The injector temperature was fixed at 280 °C and splitless mode was used with helium carrier gas. The ion source was electron ionization and the MS source temperature was set at 230 °C. The injection volume was 1.0 µL.

Propolis extract content analysis

The mass-spectrometer measurements were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer API 4000 QTRAP (Applied Biosystems, Darmstadt, Germany) with electrospray ionization (ESI). LC separations were performed in a C18 analytical column (Gemini® 5 µm particle size, 110 Å pore size, 50 mm x 2 mm, fully porous organo-silica LC Column) using a mobile phase consisting of 0.1% aqueous formic acid solution (phase A), and Methanol (phase B) at a flow rate of 0.3 mL min−1.The gradient profile started at 15% of B until 1.5 min; then it

went to 55% B in 0.1 min and kept until 3 min; then it went to 90% B in 0.1 min and kept until 4 min. Finally it was back to 15% B in 0.1 min.The run time for each injection was *5.5* min, the temperature of the column was 40 ◦C and the injection volume was10 µL. The mass-spectrometer was working with an electro spray ion source (ESI) in positive mode under the selected ion monitoring (SIM) or Selected Reaction Monitoring (SRM) condition including 0,70 amu width (the nebulizer pressure was 55psi, the drying gas (He) temperature was 40 ◦C, the drying gas flow was 1 mL/min and the skimmer voltage were among *20-80* V. Data acquisition was carried out with Workstation Method Builder) shown in Table 1. The ionization source parameters were: source temperature *50 ◦C;* curtain gas (nitrogen) 55 psi, ion spray voltage 5000V on needle, 600V on shield, 70V on capillary and 1800V on detector ; and GAS 1 and GAS 2 (both of them nitrogen) were set to 55 psi.

Herbal cream preparation

PEG400 was added to 115 mL of water, stirring slowly and thoroughly on a magnetic stirrer until thickening was achieved. After the desired consistency was achieved, a herbal cream was obtained by adding all additives in the amounts specified in the table 1.

Table 1. Additives in cream formulation

Additive, INCI name	**Amount***
Aqua	115 mL
Sodium Polyacrylate (and) Dicaprylyl Carbonate (and) Polyglyceryl-3 Caprate	2.5 g
PEG400	15 g
Propolis Extract, in solution form	1.5 g
Coco-Caprylate	10.5 g
Hypericum perforatum Oil	15 g
Perfume	3 g
Sodium Stearoyl Glutamate	0.45 g

* Amounts for ~150 g cream.

Microbiological analysis test

ISO21149[34], ISO16212[35], ISO18416[36], ISO22717[37] and ISO21150[38] standards were taken as reference for microbiological analysis aimed at determining the presence of microorganism in the cream. In the analysis, *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231), aerobic mesophilic microorganism and mold-yeast microorganisms were investigated. Briefly; In the determination of aerobic mesophilic microorganism and mold-yeast; 1 mL/gr sample was placed in 9 mL neutralizer and homogenized. 0.1 mL was taken from this mixture and transferred to Tryptic Soy Agar (TSA) medium containing Polysorbate 80 and Lecithin for aerophilic mesophilic determination and Saboraud 4% Dextrose Agar (SDA) for mold and yeast determination. Bacteria were incubated at 32.5 ± 2.5 °C for 48-72 hours and mold-yeast for 3-5 days at 22.5 ± 2.5 °C and colony counts were made at the end of the period. In the analysis of pathogen strains, in addition to those mentioned above, the neutralized sample mixture was enriched by incubating at 32.5 °C for 24 hours. At the end of the period, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* were incubated at TSA, *Candida albicans* SDA for 48 hours at 32.5 °C. Since the pathogen microorganisms should not be present in 1g or 1 mL of the product, it was checked for its existence.

Antimicrobial efficacy test

The antimicrobial efficacy test, also known as the challenge test, determines the effectiveness of the preservative by calculating the logarithm of the number of viable microorganisms remaining on the product on days 7, 14 and 28 after artificial contamination on the product. This analysis of propolis extracts used as a preservative in the cream was made with reference to the ISO11930:2012[39] standard. Microorganisms used to create contamination were *Pseudomonas aeruginosa* (ATCC 9027)*, Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231) and *Aspergillus brasiliensis* (ATCC 15404). Briefly; Working cultures were created by making subcultures from stock cultures. Microorganisms were suspended in diluent and calibrated to 10^6-10^7cfu/mL. Ten fold dilutions were made and bacteria were incubated

at TSA, yeast SDA and mold PDA for 32.5±2.5 C for 24-48 hours. 20 grams of propolis cream sample was placed in sterile containers, and 0.2 mL of different microorganisms were added to each container to ensure homogeneity. The inoculated containers were incubated at 22.5 ± 2.5 °C in the dark for 28 days. For the 7th day, 1 g of cream sample was taken from the containers and homogenized in 9 mL of neutralizer. The mixture, which was kept at room temperature for 30±15 minutes, was diluted to correspond to 1/10 and 1/100. This dilution was transferred to the SDA medium for TSA yeasts and the PDA for molds. The colonies in the petri dishes incubated under appropriate conditions were counted and logarithmic evaluation was done and according to the ISO11930: 2012 standard Criterion A was interpreted. The same procedures were carried out on the 14th and 28th days.

RESULTS

Hypericum perforatum oil analysis

It was determined that the oil of *Hypericum perforatum*, which was obtained from Duzce region of Turkey, contained fatty acids widely used in the cosmetics and pharmaceutical industry in the GC-MS analysis (Table2). Palmitic acid derivatives found in *Hypericum perforatum* masere oil can prevent the formation of wrinkles and delay skin aging. They also play a role in increasing water retention of the skin and moisturizing the skin. Linoleic acid, oleic acid, elaidic acid molecules are used in the cosmetic industry as solubilizer and plasticizer. Stearic acids are widely used in cosmetic formulations such as creams, lotions, eye makeup products, shampoos, and hair care auxiliary products [40,41]. Oleic acid derivatives are used as emulsifying and dissolving agents in addition to being used as excipients in pharmaceuticals and cosmetics [42]. The use of *Hypericum perforatum* oil in cream formulations can provide a supportive role in reducing scars and acne blemishes as well as moisturizing the skin.

Propolis extract analysis

Many studies have been conducted to evaluate the chemical composition and potential pharmacological activities of propolis produced by different bee species. Various solvents were used in these studies. Both the chemical composition and biological properties of propolis extracts depend greatly on the type of solvents used for extraction.

Table 2. GC-MS library scan results

Compound Name	RT (min)	% of Total
Hexadecanoicacid, methyl ester (Methylpalmitate)	13.47	4.7
Linoleicacidmethyl ester	16.27	40.8
(E)-9-Octadecenoic acidmethyl ester (Elaidicacidmethyl ester)	16.37	50.7
Octadecanoicacidmethyl ester (Stearicacid, methyl ester)	16.79	3.0
cis-11-Eicosenoic acidmethyl ester	20.57	1.0

The most commonly used solvent for the extraction of propolis is ethanol[43]. Therefore, the chemical content of the ethanolic extract in Duzce region of Turkey propolis used in the study was determined by LC-MS/MS analysis. The total phenolic content of the extract was determined as 66.096 ± 1.546 mg gallic acid equivalent (GAE) / mL sample, dry weight and flavonoid content as 13.375 ± 0.185 quercetin equivalent (QE) / mL sample. In addition to the total flavonoid content, it was determined that the caffeic acid compound with high free radical activity was found in high amounts in the propolis ethanol extract. An extract rich in phenolic components such as quercetin, p-coumaric acid, ferulic acid, trans-cinnamic acid, benzoic acid responsible for biological activity was obtained. When the analysis results were evaluated, it was clearly observed that the chemical content of the ethanolic extract would be suitable for use as a natural preservative (Table3).

Microbiological analysis test

As a result of the analysis, the total number of aerobic mesophilic microorganisms and mold-yeast in all samples was found as <10 cfu/g. Based on this analysis, it was found that the cream sample containing propolis and *Hypericum perforatum* oil were found to comply with the"Guidelines on Microbiological Control of

Cosmetic Products" (Table 4).

Antimicrobial efficacy test

Analysis of the efficacy of the preservative by creating artificial contamination corresponds to Criterion A in the ISO11930: 2012 standard and according to the results of the analysis, it has been determined that the preservative is protected against microbial reproduction. The reduction values equation (Rx = logN0 -logNx) was used for logarithmic calculations (Table 5).

Table 3. Chemicalcomposition of propolisextract

Chemicalcompounds	Mean ± SD (mg/g)
Pinostrobin	4620.00± 5.46
Kaempherol	0.00
o-coumaric	58.632 ± 4.92
m-coumaric	48.30 ± 3.86
FerulicAcid	0.00
Clorogenic	3.70 ± 1.65
SinapicAcid	20.80 ± 2.78
CaféicAcid	56.90 ± 4.68
ProtocatheuicAcid	0.00
Daidzein	106.10 ± 1.43
RosemarinicAcid	417.23 ± 9.30
SyringicAcid	269.33 ± 5.60
QuercetinHyrate	7.13 ± 8.74
Trans-Chalcone	6.21± 2.69
CAPE (caffeicacidphenethyl ester)	90.30 ± 3.45
Hesperidin	111.33 ± 0.00
(±)-Catechin	57.57 ± 7.22
Trans-3-Hydroxy-Cinamic Acid	28.63 ± 2.87
GallicAcid	235.00 ± 0.00
(±) Naringenin	51.03 ± 8.16
p-CoumaricAcid	13.60 ± 7.09
3-4 DimethoxycinnamicAcid	22.57 ± 8.10
Apigenin	91.67 ± 5.02
BenzoicAcid	28.52 ± 3.18
Trans-CinnamicAcid	6.88 ± 4.92
EllagicAcid	77.77 ± 6.00
Emodin	147.17 ± 7.51
Quercetin	777.67 ± 1.77

DISCUSSION

Preservatives are substances that prevent deterioration of cosmetics and care products and microorganism contamination [44], and preservative efficacy tests that measure antimicrobial activity in the finished state of the product are performed[39]. The use of preservatives is inevitable in order to prevent corruption in cream formulations and to keep the shelf life long. Since the side effects of synthetic additives are reported, it is important to search for substances isolated from natural products as preservatives. It is known that bee product propolis has a natural preservative effect that can replace the preservative additives used in the cosmetic industry. Therefore, in the study, it was investigated whether the contents of propolis samples are suitable for use as a preservative. The chemical content of the ethanol extract of propolis samples collected as a result of beekeeping activities in Duzce region of Turkey were determined. The rich content of phenolic compounds known to be responsible for the preservative effect of propolis strengthened the idea that it could be suitable for use as a preservative in cream formulations. In addition, the moisturizing oil used in cream formulations was obtained from the *Hypericum perforatum* plant, which grows naturally in Duzce region of Turkey. In the content analysis of the oil used, it was determined that fatty acids, which are frequently used as moisturizing, plasticizing and dissolving agents in the cosmetic industry, were present. Since the use of *Hypericum perforatum* oil in the treatment of wounds and burns in traditional treatments has been known from past to present, it was thought that it could contribute to the healing of wounds and acne scars as well as its moisturizing effect. Preservative effectiveness tests of the prepared herbal cream were carried out. The most used preservative in cosmetics is paraben derivatives. In the preservative activity study with paraben derivatives; when two different paraben derivatives are used in the same combination, the log cfu/mL of most bacteria is <1 on the fourteenth day, <1 was found on the twenty-first day whensoever used alone. Log cfu/mL of yeast and mold; as using a single derivative, it was <1 on the twenty-first day, while in the use of two paraben derivatives, <1 was found mostly on the fourteenth day. In the same

study, the preservative activity of isothiazolinones derivatives was investigated, it had an effect on *Staphylococcus aureus* and *Escherichia coli* on the fourteenth day (log cfu/mL <1), and *Pseudomonas aeruginosa* had an effect on the twenty-first day (log cfu/mL <1). On the other hand, it was not effective on yeast and mold species in the twenty-eight day test[45].

Table 4. Microbiological analysis test results of cream sample

Microorganisms	Unit	Result	Standart No	Limit values
Total aerobic mesophilic microorganism	cfu/g	<10	ISO21149	<100
*Staphylococcus aureus*ATCC 6538	cfu/g	Negative	ISO21149	Negative
Pseudomonas aeruginosa ATCC 9027	cfu/g	Negative	ISO22717	Negative
Escherichia coli ATCC 8739	cfu/g	Negative	ISO21150	Negative
*Candida albicans*ATCC 10231	cfu/g	Negative	ISO18416	Negative
Mold and yeast	cfu/g	<10	ISO16212	<100

Sample of propolis used in our study reduced log cfu/mL values of all microorganisms below <1 on the fourteenth day. Our data show that propolis can be as effective as paraben derivatives and can be better than isothiazolinones derivatives. The results of our analysis have shown that propolis can be used as a preservative thanks to its antimicrobial activity and naturalness.

Table 5. Antimicrobial efficacy data of the cream sample containing propolis ethanol extract

Microorganisms	0 hours		7th day			14th day	28th day
	cfu/g	log cfu/g	cfu/g	log cfu/g	Log reduction	cfu/g	cfu/g
Staphylococcus aureus (ATCC 6538)	2,60E+07	7,41	3,00E+03	3,5	3,94	<10	<10
Pseudomonas aeruginosa (ATCC 9027)	2,50E+07	7,40	2,00E+03	3,3	4,10	<10	<10
Escherichia coli (ATCC 8739)	2,40E+07	7,38	4,00E+03	3,6	3,78	<10	<10
Candida albicans (ATCC 10231)	1,60E+06	6,20	2,00E+03	3,3	2,90	<10	<10
Aspergillus brasiliensis (ATCC 15404)	2,00E+05	5,30	2,00E+02	2,3	3,00	<10	<10

CONCLUSION

In this study, the total phenolic content of Propolis ethanol extract was determined as 66.096 ± 1.546 mg gallic acid equivalent (GAE)/mL sample, dry weight and flavonoid content as 13.375 ± 0.185 quercetin equivalent (QE)/mL sample. An extract rich in phenolic components such as quercetin, p-coumaric acid, ferulic acid, trans-cinnamic acid, benzoic acid was obtained. Component analysis clearly demonstrated that propolis ethanolic extract could show protective activity. The presence in the GC-MS analysis of *Hypericum perforatum* oil, which contains fatty acids widely used in the cosmetics and pharmaceutical industries, showed that the oil can be used safely in cream formulations. The results of microbiological analysis and antimicrobial efficacy tests of creams prepared using *Hypericum perforatum* and propolis clearly revealed that the obtained propolis extract provided protective efficacy. Thickener property of PEG400, used to provide homogeneous distribution, reduced the need for extra thickener additives.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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