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Lavender Volatile Oil: A New Solvent for Propolis Extraction, Chemical Composition, Antioxidant Activity and Cytotoxicity on T98G Glioblastoma Cell Line

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Abstract: Glioblastoma is the most aggressive and common brain tumor. The survival of the patient suffering from glioblastoma is not so long. Recently, natural products including propolis have been reported as chemo/radiosensitizers in glioblastoma treatment. Propolis is a resinous complex collected from different parts of medicinal plants. This complex structured bee product can be used after extraction of its biologically active compounds and ethanol is reported to be the best solvent. There is an urgent need to find out new, healthier, and compatible administration tools of propolis intake, especially for cancer patients. The main aim of this study is to find out the solubility of propolis in lavender volatile oil. Total phenolic content and antioxidant activity of new propolis extract was determined. Cytotoxic activity of the obtained extract was also tested on T98G glioblastoma cell lines *in vitro*. Results showed that the total phenolic content of new propolis extract was determined as 10.16 mg GAE/mL. DPPH radical scavenging activity (SC_{50}) was found as 0.34 ± 0.012 mg/mL. The cytotoxic effects appeared with concentrations of 100 µg/ml and more ($p<0.01$). It could be concluded that lavender essential oil is as efficient solvent as ethanol and might be a new administration tool of propolis, especially for cancer patients.

Key words: Lavender oil, propolis extraction, antioxidant activity, glioblastoma, cytotoxicity.

Introduction

Glioblastoma is the most aggressive and common brain tumor that might happen at any age effecting men 1.5 times more than women. The survival rate of the patient suffering from glioblastoma is reported as one year after diagnosis. Surgery and adjuvant chemoradiation therapy (CRT) are two common treatments but the survival rate is still very poor [1]. Recently, natural products including propolis have been reported as chemo/radiosensitizers in glioblastoma treatment [2].

Propolis is a resinous complex collected from different parts of medicinal plants. This complex structured bee product mainly composed of volatile compounds (mono and sesquiterpenes), phenolic compounds (phenolic acids and flavonoids) and waxes (plant wax and beeswax) [3]. Propolis possesses many biological activities like anti-microbial, anticancer, anti-inflammatory, and enzyme inhibition properties because of its high phenolic content. Traditionally, propolis has been used as a remedy all around the world. However, raw propolis is not suitable for daily consumption because of its resinous nature. Propolis should be extracted to convert it into consumable form and 70 % of ethanol-water mixture is the first choice for this purpose. Therefore, some of the most common formulations containing all biologically active constituents of propolis are based on a

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solvent mixture of water and ethyl alcohol, the so-called tincture [4]. Many papers are describing the cancerous nature of ethanol in literature [5]. Daily consumption of the tincture leads to ethanol intake regularly and this situation is one of the factors limiting the usage of propolis tincture health-wise. There is an urgent need to find out new, healthier, and compatible administration tools of propolis intake, especially for cancer patients.

Volatile oils have been used either for cure or the prevention of certain diseases in aromatherapy applications. Lavender volatile oil is composed of mainly linalool and linalyl acetate. Antimicrobial, anti-inflammatory, anticancer, etc. activity of both lavender volatile oil and propolis has been reported in literature [6,7,8]. The main aim of this study is to clarify for the first time the possibility of lavender volatile oil as a solvent for propolis extraction. Cytotoxic activity of the obtained extract was also tested on the T98G glioblastoma cell line *in vitro*. At the same time, the mouse fibroblastic cell line (L929) was also tested with the same substances as the negative control.

Materials and methods

Materials

Propolis sample was collected with traps from local beekeepers in Bilecik city Turkey in summer 2019. N-Methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA) was obtained from Cova Chem, LLC. $FeSO_4.7H_2O$, methanol, Gallic acid, ethanol were purchased from Sigma Aldrich, USA. All other reagents were analytical grade. Lavender volatile oil produced by steam distillation technique was purchased from a local supplier.

Methods

Preparation of propolis extracts

The extraction of propolis with lavender volatile oil and ethanol was carried out by a simple maceration technique separately. 1:10 (g/v) ratio was used for both of the solvent. Frozen propolis sample was powdered by grinding and 10 g of this fine powder was mixed 100 mL of either with Lavender volatile oil or ethanol. Extraction was carried out for 48 h on a magnetic stirrer under constant stirring at 150 rpm. Finally, mixtures were filtered separately and filtrates were labeled as lavender oil propolis extract (LOPE), and ethanol propolis extract (EPE).

Determination of total phenolic and flavonoid content

Total phenolic content of lavender oil, lavender oil propolis extract (LOPE), and ethanol propolis extract (EPE) were determined by using Folin-Ciocalteu method [9], Gallic acid was used as standard. Results were expressed as mg GAE/mL. The total flavonoid content of the samples was determined by using aluminum chloride method [10], quercetin was used as standard. Results were expressed as mg QE/mL.

Determination of antioxidant activity

Antioxidant activity of lavender oil, lavender oil propolis extract (LOPE), and ethanol propolis extract (EPE) were measured by using ferric reducing antioxidant power (FRAP) [11] and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging method [12]. Results were expressed as µM Trolox equivalent/ g sample for FRAP and SC_{50} (mg/mL) for DPPH respectively.

GC/MS analysis of the samples

The main chemical composition of lavender oil, lavender oil propolis extract (LOPE), and ethanol propolis extract (EPE) were determined with Gas chromatography coupled with mass spectrometry. Derivatization of propolis extracts was carried out by using N-Methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA). Shortly, propolis extracts were dried by using a rotary evaporator, and 5 mg of dried residue was mixed with 50 µL of dry pyridine and 75 µL of MSTFA. This reaction mixture was heated at 80°C for 20 min.

GC-MS analysis was applied with an Agilent 7890A GC system equipped with an HP5-MS capillary column (30 m x 0.25 mm x 0.5 mm). The oven temperature was programmed from 75 to 325°C at a rate of 5°C/min, and a 15 min hold at 325°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:50, the injector temperature 300°C, and the ionization voltage 70 eV[13].

The identification of the compounds was performed using Wiley as commercial libraries.

Cell lines study

The human glioma cell line T98G and mouse fibroblastic cell line L929 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) / Ham's F12 (Biochrom AG, Berlin, Germany) supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), and 1% penicillin/streptomycin at 37°C in a humidified atmosphere (5 % CO_2 in air). The culture medium was refreshed every two days and sub-cultured till the experimental protocol was applied. The reason why the T98G cell line was employed was that it is a human-grade IV glioma cell [14]. We selected them because they are the most extensively employed ones in related studies [15]. Also, the reason why the L929 cell line was preferred to use in this *in vitro* experiment is that it is widely used for cytotoxicity tests due to their unlimited life span and their ability to multiply rapidly [16]. Raw propolis was extracted in ethanol and lavender volatile oil to obtain stock solutions. Cultures of malignant glioma cells were treated with increasing concentrations of lavender oil propolis extract (LOPE) and ethanol propolis extract (EPE). The non-treated group served as the control group. Ethanol was used for preparing extracts in different concentrations and the final concentrations of ethanol in the medium did not exceed 1 %.

Cell viability assays

Cultures of human glioma cells and mouse fibroblastic cells were treated with ethanol alone and with lavender oil propolis extract (LOPE) at a concentration of 10, 50, 100, and 200 µg/ml. Cells viability were assessed by 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. It is a standardized cellular viability test, known as a colorimetric method that assesses the ability of viable cells. Following treatment of cells with substances, well-plates were cultured at 37°C and 5 % CO_2. For assessment of cellular viability, at the end of the designed incubation periods, the culture medium was replaced with 200 µL medium containing 10 % MTT, and plates were incubated for four hours at 37°C. To dissolve the formazan crystals, the solution was removed and replaced with isopropyl alcohol. The absorbance was measured on a spectrophotometer microplate reader (µQuantTM, BioTek® Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm. Assays were performed at least three times and represented as the mean value. Cell cultures were observed every day by light microscopy, and viability test was performed at 24, 48, and 72 h post-treatment with lavender oil propolis extract (LOPE). Percentage cellular viability was calculated with the formula given below.

Cellular Viability (%) = (Treated Group Absorbance) / (Control Group Absorbance) x 100

Results and discussion

Determination of total phenolic and flavonoid content

The total phenolic content of extracts was determined as 11.99 and 10.16 mg GAE/mL for EPE and LOPE respectively (Table 1). Keskin and Kolayl [17] determined the total phenolic and flavonoid content of some Anatolian propolis samples. They reported that the total phenolic content of ethanol extracts prepared with

Table 1. Total phenolic content of extracts

Sample	Total phenolic content mg GAE/ mL	Total flavonoid content mg QE/ mL
EPE	11.99 ± 0.08	2.66 ± 0.05
LOPE	10.16 ± 0.07	1.85 ± 0.08
LO	0.125 ± 0.02	nd

Results are mean value of three separete measurements with standard deviations
EPE: Ethanol propolis extract; LOPE: Lavander oil propolis extract; LO: Lavander oil; nd: Not determined

Anatolian propolis samples ranged between 16.13 to 178.34 mg GAE/mL[17]. Can *et. al* [18] studied antioxidant properties of some Azerbaijan propolis samples and they reported that the total phenolic content of the samples were between 10.94 and 79.23 mg GAE/g [18]. The total phenolic content of propolis samples differs depending on the botanical origin [19]. Lavender volatile oil used in the present study is composed mainly of linalool and linalyl acetate. Chemically the structure of linalool is an analogue of primary alcohols. Balsam, ethanol-soluble fraction, contains the main biologically active components of propolis. Many solvents have been tested for the extraction of propolis balsam and 70 % ethanol-water mixture is reported to be the best one. Other solvents like glycerol and glycols are reported to be as second choice for extraction of propolis balsam. Lavender volatile oil has been tested for the first time for propolis extraction. It is clear from the table that lavender volatile oil is as efficient as ethanol for the extraction of propolis phenolic compounds when compared to the total phenolic content. This result could be explained by the structural similarity of linalool and ethanol.

Determination of antioxidant activity

Ferric Reducing Activity (FRAP) and DPPH free radical scavenging activity tests are commonly used for the determination of the antioxidant potential of natural products. Results for antioxidant activity of the extract were summarized in Table 2. DPPH radical scavenging activity of lavender volatile oil was reported with IC_{50} value as 31.3 mg/mL. Propolis and volatile oils are separately reported as a good radical scavenger in literature because of their ability for donating the hydrogen to the DPPH radical [20]. DPPH radical scavenging activity of lavender volatile oil extracted by using supercritical CO_2 extraction was reported as 73 % when 40 μL of oil was reacted with 0.4 ml of 0.5 mM DPPH in absolute ethanol [21]. Many papers were reporting antioxidant activity of propolis in literature as well [13,17,18]. Better antioxidant activity of new propolis extract (LOPE) could be explained by the synergism of lavender oil and propolis.

GC/MS analysis of the samples

The main chemical composition of lavender essential oil was determined as linalool (44.56 %) and linalyl acetate (26.36 %) (Table 3). Similar results were reported in the literature [22]. Determination of the phenolic and flavonoid content of a propolis extract is a great importance for establishing integrity between propolis extract and its biological activity. Determined chemical compounds in propolis extracts were listed in Table 4. The findings of the present study showed that raw propolis samples contained certain types of phenolic acids and flavonoids. It was reported that some propolis samples from Anatolia were rich in some phenolic acids like cinnamic acid, caffeic acid, ferulic acid, and p-hydroxybenzoic acid and their esters. It was also mentioned in earlier works that Anatolian propolis were rich in certain flavonoids namely pinobanksin, pinocembrin, galangin, and chrysin [3,19,23]. Our findings clearly showed that the propolis sample used in this study showed good similarity with poplar type propolis [19]. Determination of these phenolic acids and flavonoids both in ethanol propolis extract and lavender oil propolis extract means that lavender oil is as efficient as ethanol for the extraction of propolis.

Table 2. Antioxidant activity of lavander oil and propolis extracts

Sample	DPPH SC_{50} (μg/mL)	FRAP (μM TE/g)
EPE	19.91 ± 0.072	350.79 ± 0.07
LO	86.24 ± 0.054	470.26 ± 0.06
LOPE	5.48 ± 0.012	720.28 ± 0.08

Results are mean value of three separate measurements wits standard deviations
EPE: Ethanol propolis Extract; LO: Lavander Oil; LOPE: Lavander Oil Propolis Extract

Table 3. Main chemical composition of lavender essential oil

No.	Component	RI Experimental	RI Literature	Area % ± SD
	Monoterpenes			
1	α-Pinene	935	936	0.56 ± 0.09
2	Camphene	944	950	0.46 ± 0.08
3	Limonene	1028	1029	2.16 ± 0.05
4	o-Cymene	1038	1041	1.06 ± 0.07
	Terpenoids			
5	Linalool	1103	1099	44.56 ± 0.14
6	Camphor	1138	1143	1.16 ± 0.07
7	Linalyl acetate	1161	1255	26.36 ± 0.11
8	Borneol	1269	1166	1.76 ± 0.09
9	Lavendulyl acetate	1285	1289	2.26 ± 0.06
10	Eucalyptol	1024	1031	5.60 ± 0.12
	Sesquiterpene			
11	Caryophyllene	1412	1420	4.76 ± 0.08
	Total			90.07 ± 0.10

Table 4. Chemical composition of propolis extracts

	% of TIC	
Components	**EPE**	**LOPE**
Phenolic acids		
Dihydrocinnamic acid	0.60	0.45
Cinnamic acid	1.10	0.98
p-Hydroxybenzoic acid	0.15	0.16
Vanillic acid	0.14	0.12
p-Coumaric acid	1.15	1.02
Caffeic acid	1.80	1.68
Ferulic acid	0.98	0.86
Phenolic acid esters		
Cinnamyl cinnamate	0.34	0.32
Benzyl caffeate	3.96	3.88
Benzyl ferulate	0.50	0.48
Caffeic acid phenethyl ester (CAPE)	2.45	2.10
Ferulic acid phenethyl ester	0.98	0.84
Cinnamyl *p*-coumarate	0.62	0.56
Cinnamyl caffeate	2.90	2.40
Cinnamyl ferulate	1.86	1.74
Chalcones		
Pinobanksin chalcone	1.56	1.25
Pinocembrin chalcone	14.02	13.98
Pinostrobin chalcone	0.85	0.76
Flavonoids		
Pinobanksin	3.50	3.40
Pinocembrin	6.52	6.12
Pinostrobin	0.32	0.28
Galangin	12.10	11.80
Chrysin	8.20	7.80
Tectochrysin	0.70	0.62

Cell line study and viability assays

To investigate the sensitivity of L929 and T98G cell lines to propolis extract cells were seeded and were exposed to extracts (10, 50, 100, and 200 µg/ml) after 24 h of incubation. The results indicated that only the T98G cell line was sensitive to treatment with propolis extract in a dose-dependent manner (Table 6). IC_{50} values were determined by plotting a log concentrations and % cell viability graph. IC_{50} represents the concentration of µg/mL required for 50 % inhibition of cell growth compared to control cells (Table 5). Propolis extract (extracted with 70 % ethanol) had no adverse effect on the non-cancer mouse fibroblast cell line. When cytotoxicity of propolis extract in different doses was examined, up to 100 µg/ml concentration, a decrease in viability was not significant ($p<0.0001$). The cytotoxic effects appeared with concentrations of 100 µg/ml and more ($p<0.01$) (Table 5). It has been found that especially with all concentrations of LOPE induced cytotoxicity 48 h post-treatment. Collectively, all substance reduces cell proliferation and induces cytotoxicity in glioma cells in different concentrations than in normal mouse fibroblasts. In a study, it was reported that propolis extract obtained with ethanol exhibited inhibition activity on the proliferation of U87MG cells [24]. Another study reported by Atabay *et. al*.[25] revealed that propolis samples obtained from Giresun city, Turkey showed anti-proliferation activity on glioblastoma cells. The apoptotic cell population is reported to be significantly greater when exposed to higher propolis concentrations [25]. Sienkiewicz *et. al*.[7] reported the effects of essential oils (cinnamon, geranium, and lavender) on the viability of human microvascular endothelial cells (HMEC-1) and glioblastoma cell line (T98G). Among the tested oils, lavender oil is reported to exhibit the lowest cytotoxicity towards T98 cells, with the IC_{50} values of lavender against HMEC-1 and T98G cells being 5.15 µL/mL and 2.27 µL/mL, respectively [7]. The selectivity index which represents the ratio of IC_{50} values of EPE, LOPE, and LO for normal cells (L929) to IC_{50} values for brain cancer cells (T98G) indicates that new propolis extract specifically effects cancer cells much more than normal cells exhibiting selective toxicity (2.1-fold).

Conclusions

Lavender oil has been tested for propolis

Table 5. IC_{50} (µg/mL) values and selectivity index of the samples

Sample	T98G Cell line	L929 Cell line	Selectivity Index
EPE	19.53±0.13	85.02±0.25	4.3
LOPE	47.05±0.04	100.84±0.16	2.1
LO	12.57±0.01	89.92±0.03	7.1

EPE: Ethanol propolis extract; LOPE: Lavander oil propolis extract; LO: Lavander oil (n:3)

Table 6. The mean T98G cell viabilities (%) after 48 hour treatment with substances

	Cell viability % (Mean ± SD)			
Concentrations	EPE	LO	LOPE	P*
10 µg/ml	0.090±0.01	0.045±0.01*	0.054±0.00*	0.014
50 µg/ml	0.059±0.03*	0.056±0.02*	0.053±0.04*	0.004
100 µg/ml	0.096±0.01	0.065±0.01	0.052±0.00*	0.001
200 µg/ml	0.070±0.02	0.75±0.03	0.050±0.00*	0.011

*Bonferroni post-test

EPE: Ethanol propolis extract; LO: Lavander oil; LOPE: Lavander oil propolis extract

extraction for the first time up to now. Findings of the present study clearly show that lavender oil is a good extractor of propolis balsam which contains biologically active components. Moreover, the obtained extract exhibited good *in vitro* cytotoxic activity on the glioblastoma cell line. It can be concluded that lavender volatile oil propolis extract could be an alternative in the treatment of glioblastoma.

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