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Original article

Characterization of Propolis Extracts Prepared Using Different Solvents at the Different Concentrations

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Summary: Propolis is an important bee product thanks to having many biological activities such as antibacterial, antiviral, antifungal. However, it has a complex structure as it contains many components such as beeswax, balm, pollen, flavonoids, and phenolic acids. Useful components of this structure must be dissolved in an effective and also a reliable solvent in order to be released. In this study, propolis samples were collected from different regions of Turkey and these samples dissolved in various solvents with different concentrations (70% glycol was used for water-soluble propolis extracts of 10% and 15%, and 70% ethanol was used for ethanol extracts of 20% and 30%) for the preparing extracts. Then, in these extracts, dry matter, total phenolic, total flavonoid and antioxidant capacity were determined and compared to the each other. A statistically significant difference was found between the results of all the concentrations examined in this study ($p<0.01$). In addition, as a result of the results obtained, 20% ethanol extract (70% v/v ethanol) and 15% glycol propolis extract (70% v/v glycol) are statistically similar; and 30% ethanol propolis extract (70% v/v ethanol) was found to contain the most bioactive components ($p<0.01$). With this study, it has been revealed that propolis, which differs in botanical origin and component content, can be prepared in certain standards depending on the solvent type and concentrations. Due to the fact that there is very little study and literature knowledge on this subject within our knowledge, these results can be useful of the basic standardization of the propolis.

Keywords: antioxidant; ethanol extract; flavonoid; glycol extract; phenolic; propolis

Introduction

Propolis is a bee product produced by honeybees (*Apis mellifera* L.) by processing resinous substances collected from plant buds and secretions. The content and color of raw propolis vary according to the plant source and environmental conditions (hive, season, region). Generally, it consists 50% balsam, 30% beeswax, 10% essential oils and 5% pollen and color can be brown, red or green (1, 2). The main groups of compounds in propolis are phenolic acids and esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids and steroids (3). These compounds, especially phenolic acids and flavonoids show a wide variety of biological and pharmacological activities (4).

It is not appropriate to consume the raw form of such useful product propolis, so raw propolis should be turned to the extracted form. Propolis extraction methods and solvents are very important for the reveal of active compounds in the content of propolis. It is generally reported that the active solvent of propolis is ethanol and 70% ethanol reveals phenolic and flavonoids,

which are important components of propolis. Moreover, since the content of propolis varies according to the plant source, the ethanol concentration is effective in the reveal of the active ingredients of propolis (3).

Propolis extracts need to be standardized in order to show similar or close activity before exposing for sale on the market. It is possible to make simple standardization of extracts prepared by the analysis made with known methods such as antioxidant capacity, total phenolic amount determination (5-7). Therefore, in this study, % dry matter, total phenolics, total flavonoids and antioxidant capacity were analyzed and compared at the 10% and 15% glycol (70% v/v glycol) propolis extract, 20% and 30% ethanol (70% v/v ethanol) propolis extract samples. The best concentration among the current forms was determined, thus the simple standardization was performed for quality control. This study is thought to contribute to the national and international propolis standard studies.

Material and Methods

Collecting of Propolis Samples

Propolis samples were obtained from beekeepers producing with a contract apiculture model from 58 different provinces of Turkey in 2019. Propolis traps placed in the hives in spring season were harvested end of the summer. Traps were kept in the freezer and were removed from the freezer while preparing propolis extracts.

Dry Matter Analysis

Dry matter analyzes were performed at 105°C with the Precisa XM50 infrared heater moisture analyzer according to the modified AOAC 934.01 method (8).

Preparation of Extracts from Raw Propolis

Raw propolis were kept in the -18 °C freezer overnight and then ground into powder. 70% glycol for water-soluble propolis extracts of 10% and 15% and 70% ethanol were used for ethanol extracts of 20% and 30%. The prepared extracts were shaken on a horizontal shaker at room temperature for 6 days and after centrifugation at 2700 g for 10 minutes, these were analyzed (9).

Determination of Total Phenolic Content

Total phenolic content was found by modifying the Meda et al. (2005) method (10). According to this method, the working curve was prepared using varying concentrations of gallic acid standard for calibration and the results were expressed as mg Gallic Acid Equivalent (GAE) per mL.

Determination of Total Flavonoid Content

Total flavonoid content was found by modifying the Dewanto et al. (2002) method (11). According to this method, the working curve was prepared using varying concentrations of catechin standard for calibration and the results were expressed as mg Catechin Equivalent (CE) per mL.

Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

Cupric Ion Reducing Antioxidant Capacity (CUPRAC) was detected according to the Apak et al. (2004) (12). According to this method, the working curve was prepared using varying concentrations of trolox standard for calibration and the results were expressed as mg Trolox Equivalent (TE) per mL.

Statistical Analysis

All parameters tested for the normal distribution accordingly method and extract differences. Accordingly, method differences were analyzed by non-parametric Kruskal Wallis test for dry matter, total phenolic and total flavonoid parameters. The method differences for the CUPRAC parameter were investigated using One-way ANOVA with application the logarithmic transformation. Moreover, the extract differences for the parameters that have normal distribution or normalized by transformation were investigated using One-way ANOVA in each method. For these purposes, the logarithmic

transformation was applied to total phenolic and total flavonoid in 10% and 15% glycol (70% v/v glycol) propolis extract, respectively. Non-parametric Kruskal Wallis test was used for dry matter and CUPRAC in 10% (70% v/v glycol) propolis extract, and 30% (70% v/v ethanol), CUPRAC in 15% (70% v/v glycol) concentrations. The differences between parameters were described by mean rank, mean and standard deviation obtained from Kruskal Wallis test; mean and standard errors obtained from ANOVA. Duncan multiple comparison tests were applied for comparing differences between extract means for One-way ANOVA and Dunn's test for Kruskal-Wallis test. All statistical tests were performed at 5% level of significance by IBM SPSS statistics v24.

Results and Discussion

Raw propolis is not a natural mixture that can be easily consumed due to the resin and wax-like substances in its structure. For this reason, the biologically active components in its structure are revealed by the extraction method applied and, solvents such as ethanol, glycerol, polyethylene/polypropylene glycol, glycerol, water are used (13). Propolis has an additional food role as it is a good source of phenolics.

In this study, the average amount of dry matter ranged was determined 13.07%, 17.94%, 24.58%, and 37.31% respectively; the average amount of total phenolic content ranged between 48.15, 55.96, 59.34, and 79.76 mg GAE.mL-1; the average amount of total flavonoid ranged content was revealed 26.21, 36.91, 37.97, and 53.68 mg CatE.mL-1 respectively and the average antioxidant capacity (CUPRAC) ranged between 116.90, 151.94, 165.16, and 236.49 mgTE. mL-1 respectively (Table 1). Statistically, normal distribution test was applied to dry matter, total phenolic, total flavonoid and CUPRAC variables and it was determined that the data subject to the research did not show normal distribution. Logarithmic transformation was applied to the CUPRAC variable and the difference between the methods was examined by one-way analysis of variance (ANOVA). The difference between the methods for dry matter, total phenolic and total flavonoid variables were investigated with the Kruskal-Wallis test.

Beside this, we compare the all properties of the different propolis concentrations (Table 2-5). According to these, the 10%, 15% glycol propolis extracts and 20%, 30% ethanol propolis extracts were found statistically different based on the dry Matter, total phenolic, total flavonoid and CUPRAC parameters ($p<0.05$). Moreover all the extracts were significantly different related to mentioned parameters ($p<0.05$).

Dry matter is also referred to as dry residue free from volatile substances. It was determined that the dry matter amount of hydroalcoholic propolis extracts varied between 5.50% and 9.30%. It is reported that total phenolic content and flavonoid content of propolis samples varied between 23.44 and 53.91 mgGAE.g-1 propolis, total flavonoids 11.23 and 15.88 mgQE.g-1 propolis, respectively (14). It is reported that the amount of total phenolic content of propolis samples obtained from Brazil, China and Uruguay varies between 10.10% and 28.60%, and the total amount of flavonoid substance varies between 3.00% and 6.60% (15). It is reported that the amount of total phenolic content in the 70% ethanol extracted propolis samples from Brazil ranged between 8.8% and 13.7%, while the amount of flavonoids was minimum 0.35% and 2.7% (16). Total phenolic content of different commercial ethanolic propolis extracts varied between 10.48 and 77.68 mg GAE/mL (7). In order to carry out a simple standardization study, the amount of total phenolic and flavonoid content of propolis samples collected from different regions of Bulgaria was reported to vary between 11.2-41.9% and 2.9-13.5%, respectively (17). It is stated that cupric ion reducing antioxidant capacity (CUPRAC) of propolis samples varied between 0.27 ± 0.08 and 0.40 ± 0.09 mmol Trolox.g-1 (18). It is determined that CUPRAC of propolis samples collected from different regions were 12404 ± 64 to 35721 ± 57 μM TE.100 mL-1 (19). It is investigated the effect of different solvents on propolis in their study. According to this study, total phenolic and flavonoid contents ranged between 0.81 ± 0.16 and 8.97 ± 0.25 EGA mg.g-1 and from 0.57 ± 0.01 to 3.53 ± 0.84 EQ mg.g-1 respectively (20). All data obtained as a result of the study were found to be compatible with the literature.

As a result of the analyzes, it has been determined that 30% ethanolic propolis extract has higher dry matter and is the richest in terms of total phenolic,

Table 1. The mean, mean rank and standart errors of 10% and 15% (70% v/v glycol) glycol propolis extract, 20% and 30% (70% v/v ethanol) ethanolic propolis extract samples

	Concentrations (%)	N	Mean	Std. Deviation	Mean Rank	*p*
Dry Matter	10	36	13.07	2.87	24.89 [d]	**
	15	57	17.94	2.35	62.37 [c]	
	20	33	24.58	3.13	107.76 [b]	
	30	48	37.31	5.99	150.38 [a]	
Total Phenolic	10	36	48.15	9.52	40.78 [c]	**
	15	57	55.96	13.67	70.68 [b]	
	20	33	59.34	6.36	85.44 [b]	
	30	48	79.76	9.53	143.94 [a]	
Total Flavonoid	10	36	26.21	7.08	30.96 [c]	**
	15	57	36.91	9.05	76.59 [b]	
	20	33	37.97	4.83	83.30 [b]	
	30	48	53.68	6.21	6.21	

a,b,c,d: Letters within same column show significant differences between methods (p<0.05).
**$P < 0.01$

	Concentrations (%)	N	Mean	Std. Deviation	Std. Error	*p*
Antioxidant Capacity (CUPRAC)	10	36	116.90	16.01	2.67 [d]	**
	15	57	151.94	23.48	3.11 [c]	
	20	33	165.16	13.46	2.34 [b]	
	30	48	236.49	37.79	5.46 [a]	

a,b,c,d: Letters within same column show significant differences between methods (p<0.05).
**$P < 0.01$

Table 2. The mean, mean rank and standart errors of 10% (70% v/v glycol) glycol propolis extracts

	Dry Matter			Total Phenolic		Total Flavonoid		CUPRAC		
Extract	**Mean Rank**	**Mean**	**Std. Dev.**	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean Rank**	**Mean**	**Std. Dev.**
1	14.00 [cd]	11.53	0.81	50.20	1.62 [bcd]	35.03	1.01 [b]	3.83 [ac]	99.40	1.81
2	16.00 [cd]	11.77	0.55	45.87	1.56 [cd]	17.90	0.55 [f]	11.83 [cd]	104.57	3.80
3	15.67 [cd]	11.70	0.90	54.33	1.09 [b]	24.53	0.69 [de]	29.33 [cd]	131.67	1.79
4	4.50 [ac]	10.03	0.59	51.77	0.78 [bc]	20.40	0.61 [ef]	23.00 [cd]	124.03	2.15
5	22.50 [cd]	13.10	1.10	48.27	1.55 [bcd]	18.10	1.13 [f]	27.33 [cd]	130.23	2.06
6	10.00 [cd]	11.03	0.40	38.60	1.67 [f]	28.97	1.16 [c]	10.67 [cd]	104.57	1.40
7	29.00 [cd]	16.30	0.44	47.83	1.36 [bcd]	23.10	1.63 [de]	30.33 [cd]	132.77	3.26
8	6.00 [ac]	10.37	0.49	40.73	0.87 [ef]	26.80	1.68 [cd]	3.83 [ac]	99.97	2.80
9	25.17 [cd]	13.73	0.60	73.90	1.87 [a]	42.13	0.49 [a]	35.00 [bd]	150.17	3.29
10	33.50 [bd]	17.93	0.97	44.50	1.16 [d]	29.77	1.96 [c]	12.67 [cd]	105.87	1.29
11	33.50 [bd]	18.17	0.95	37.63	0.58 [f]	23.90	2.31 [de]	19.67 [cd]	112.33	1.34
12	12.17 [cd]	11.20	1.44	44.13	1.83 [de]	23.87	0.85 [de]	14.50 [cd]	107.20	3.24
p	**			**		**		**		

a,b,c,d,e,f: Letters within same column show significant differences between methods (p<0.05).
**$P < 0.01$

Table 3. The mean, mean rank and standart errors of 15% (70% v/v glycol) glycol propolis extracts

Dry Matter			Total Phenolic		Total Flavonoid		CUPRAC		
Extract	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean Rank**	**Mean**	**Std. Dev.**
1	19.00	0.58 ^{cd}	48.60	1.60 ^{e}	36.90	1.57 ^{d}	38.00 ^{cd}	159.27	0.49
2	19.80	0.58 ^{bcd}	54.43	1.60 ^{d}	44.67	1.45 ^{c}	47.00 ^{cd}	171.33	3.03
3	22.87	0.58 ^{a}	58.97	1.60 ^{d}	52.50	1.78 ^{ab}	13.00 ^{cd}	133.03	1.90
4	18.93	0.58 ^{cd}	55.07	1.60 ^{d}	54.40	1.33 ^{a}	54.67 ^{bd}	202.37	3.46
5	19.43	0.58 ^{bcd}	72.90	1.60 ^{b}	46.83	0.86 ^{bc}	43.33 ^{cd}	164.90	2.18
6	15.33	0.58 ^{gh}	69.83	1.60 ^{b}	28.93	1.16 ^{ghi}	41.67 ^{cd}	163.60	2.57
7	17.00	0.58 ^{efg}	86.43	1.60 ^{a}	36.07	1.67 ^{d}	54.33 ^{bd}	201.63	3.65
8	15.73	0.58 ^{gh}	47.33	1.60 ^{e}	23.47	2.39 ^{j}	9.33 ^{cd}	131.03	1.27
9	17.93	0.58 ^{def}	54.73	1.60 ^{d}	28.60	0.61 ^{hi}	28.83 ^{cd}	143.57	0.86
10	15.07	0.58 ^{h}	44.63	1.60 ^{e}	27.00	1.82 ^{i}	2.00 ^{ac}	120.40	3.60
11	16.37	0.58 ^{fgh}	68.37	1.60 ^{bc}	46.47	2.17 ^{bc}	34.67 ^{cd}	151.40	2.80
12	21.13	0.58 ^{b}	64.70	1.60 ^{c}	45.07	1.41 ^{c}	50.00 ^{cd}	181.80	2.52
13	15.17	0.58 ^{gh}	72.27	1.60 ^{b}	34.03	0.73 ^{de}	32.33 ^{cd}	147.47	2.65
14	18.50	0.58 ^{cde}	54.03	1.60 ^{d}	38.03	1.21 ^{d}	14.33 ^{cd}	132.97	0.75
15	19.40	0.58 ^{bcd}	46.90	1.60 ^{e}	31.30	1.91 ^{fgh}	5.00 ^{cd}	127.73	1.00
16	17.97	0.58 ^{def}	49.17	1.60 ^{e}	30.17	0.99 ^{fghi}	20.00 ^{cd}	138.30	2.10
17	20.00	0.58 ^{bc}	39.73	1.60 ^{f}	29.20	1.72 ^{ghi}	13.67 ^{cd}	133.67	4.12
18	15.77	0.58 ^{gh}	29.13	1.60 ^{g}	33.10	1.16 ^{def}	23.83 ^{cd}	140.90	1.30
19	15.53	0.58 ^{gh}	45.93	1.60 ^{e}	34.47	0.62 ^{de}	25.00 ^{ac}	141.57	1.25
p	**		**		**		**		

a,b,c,d,e,f,g,h,i: Letters within same column show significant differences between methods (p<0.05).

**P < 0.01

Table 4. The mean, mean rank and standart errors of 20% (70% v/v ethanol) ethanol propolis extracts

Dry Matter			Total Phenolic		Total Flavonoid		CUPRAC	
Extract	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean**	**Std. Error**
1	25.17	0.58 ^{bc}	54.77	1.57 ^{de}	42.60	1.54 ^{ab}	148.93	1.36 ^{g}
2	25.90	0.58 ^{bc}	54.53	1.57 ^{de}	44.30	1.54 ^{a}	154.50	1.36 ^{ef}
3	26.23	0.58 ^{b}	52.17	1.57 ^{f}	39.37	1.54 ^{bc}	157.67	1.36 ^{e}
4	29.77	0.58 ^{a}	68.73	1.57 ^{a}	45.47	1.54 ^{a}	188.13	1.36 ^{a}
5	28.97	0.58 ^{a}	55.13	1.57 ^{de}	36.47	1.54 ^{cde}	150.60	1.36 ^{fg}
6	24.63	0.58 ^{bc}	55.20	1.57 ^{de}	33.70	1.54 ^{de}	161.67	1.36 ^{d}
7	24.33	0.58 ^{c}	67.37	1.57 ^{ab}	33.43	1.54 ^{e}	168.97	1.36 ^{c}
8	22.13	0.58 ^{d}	67.60	1.57 ^{ab}	33.97	1.54 ^{de}	187.60	1.36 ^{a}
9	20.77	0.58 ^{de}	58.37	1.57	34.90	1.54 ^{cde}	155.47	1.36 ^{e}
10	20.03	0.58 ^{e}	55.90	1.57	38.60	1.54 ^{bcd}	173.97	1.36 ^{b}
11	22.40	0.58 ^{d}	62.97	1.57	34.90	1.54 ^{cde}	169.30	1.36 ^{c}
p	**		**		**		**	

a,b,c,d,e: Letters within same column show significant differences between methods (p<0.05).

**P < 0.01

Table 5. The mean, mean rank and standart errors of 30% (70% v/v ethanol) ethanol propolis extracts

	Dry Matter			Total Phenolic		Total Flavonoid		CUPRAC		
Extract	**Mean Rank**	**Mean**	**Std. Dev.**	**Mean**	**Std. Error**	**Mean**	**Std. Error**	**Mean Rank**	**Mean**	**Std. Dev.**
1	27.67 ^{cd}	36.53	1.46	69.67	1.73 ^{d}	54.20	1.39 ^{cde}	3.00 ^{bd}	194.53	2.00
2	30.67 ^{cd}	37.23	0.38	70.17	1.73 ^{d}	56.30	1.39 ^{c}	11.00 ^{cd}	208.87	2.17
3	15.17 ^{cd}	33.47	1.53	73.63	1.73 ^{cd}	60.50	1.39 ^{b}	20.00 ^{cd}	220.87	2.54
4	44.33 ^{cd}	48.47	1.35	74.60	1.73 ^{cd}	48.00	1.39 ^{fg}	35.00 ^{cd}	247.20	2.17
5	8.00 ^{cd}	31.83	1.42	76.17	1.73 ^{c}	46.37	1.39 ^{g}	15.67 ^{cd}	212.07	2.40
6	34.83 ^{cd}	38.57	1.05	84.40	1.73 ^{ab}	50.37	1.39 ^{efg}	8.33 ^{cd}	204.90	5.59
7	38.00 ^{cd}	41.67	2.12	81.87	1.73 ^{b}	54.20	1.39 ^{cde}	6.33 ^{bd}	200.13	9.73
8	28.00 ^{cd}	36.80	0.50	64.63	1.73 ^{e}	65.90	1.39 ^{a}	30.17 ^{cd}	233.67	2.55
9	40.67 ^{cd}	44.27	1.45	86.50	1.73 ^{ab}	51.83	1.39 ^{cdef}	29.67 ^{cd}	232.10	2.27
10	46.67 ^{bd}	50.47	1.25	76.20	1.73 ^{c}	46.57	1.39 ^{g}	12.67 ^{cd}	209.70	3.08
11	7.33 ^{cd}	31.43	1.86	81.73	1.73 ^{b}	51.63	1.39 ^{def}	42.33 ^{cd}	282.43	1.99
12	9.17 ^{cd}	31.93	1.72	86.73	1.73 ^{ab}	55.17	1.39 ^{cd}	47.00 ^{ac}	340.73	4.57
13	20.50 ^{cd}	34.83	0.49	84.10	1.73 ^{ab}	54.40	1.39 ^{cde}	42.67 ^{cd}	283.13	3.52
14	22.67 ^{cd}	35.43	0.90	73.40	1.73 ^{cd}	47.40	1.39 ^{fg}	25.83 ^{cd}	229.53	2.76
15	5.67 ^{ac}	31.00	1.61	89.40	1.73 ^{b}	50.83	1.39 ^{defg}	24.33 ^{cd}	227.53	2.80
16	12.67 ^{ac}	33.10	0.56	102.93	1.73 ^{a}	65.27	1.39 ^{a}	38.00 ^{cd}	256.50	2.07
p	**			**		**		**		

a,b,c,d,e,f,g: Letters within same column show significant differences between methods (p<0.05).

**P < 0.01

total flavonoid and antioxidant substances. In the study in which four different methods were evaluated statistically, it was determined that each group showed a similar tendency in terms of dry matter, total phenolic and flavonoid and antioxidant capacity. In addition, when the total phenolic and flavonoid amounts of 15% glycol extract and 20% ethanolic propolis extract are compared, it is seen that these have a similar trend. This indicates that two extracts prepared in different solvents and concentrations have similar properties, and that 15% glycol extract is equivalent to 20% ethanolic propolis extract.

Conclusions

In this study, the usefulness of propolis, which is an important supplementary health support in the increasingly artificial world, was investigated for the first time in various solvent concentrations. A statistically significant difference was found between methods in terms of dry matter, total phenolic and total flavonoids (p<0.05). In terms of dry matter, all concentrations differ from each other and at 30% (70% v/v ethanol) ethanol propolis extract concentration, dry matter amount, total phenolic, total flavonoid content and antioxidant capacity were higher than the other concentrations. In addition, 15% (70% v/v glycol) glycol propolis extract and 20% (70% v/v ethanol) ethanol propolis extract concentrations were found similar to each other and different from other concentrations in terms of total phenolic and total flavonoid amount (p<0.05). The highest dissolution for all parameters was at 30% (70% v/v ethanol) ethanol propolis extract concentration.

With this study, it has been revealed that propolis, which differs in botanical origin and component content, can be prepared in certain standards depending on the solvent type and concentrations. Due to the fact

that there is very little study and literature knowledge on this subject within our knowledge, these results can be useful of the basic standardization of the propolis.

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