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RESEARCH NOTE

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Antioxidant, Antimicrobial, and Cancer Cell Proliferative Inhibition Activities of Propolis

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Abstract A commercial propolis was investigated in terms of its antioxidant, antimicrobial, and antiproliferative activities. The contents of total phenol and flavonoid of propolis were 8.3 and 6.6 mg, respectively. The reducing power of the propolis increased with concentration increasing. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was shown at 82.70% in 1,000 μg/mL of the propolis. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging effect of antioxidant activity on the propolis was 35.64 g/sample. The propolis showed high antimicrobial activity against *Bacillus cereus* at all concentration of propolis. All of the cancer cell lines have 53-73% as effective growth inhibition. These results showed that the commercial propolis has potential antioxidant, antimicrobial, and cancer cell proliferative inhibition activities thus, propolis can be applied to the functional food, pharmaceutical, and cosmetic industry.

Keywords: antioxidant, antimicrobial, cancer cell proliferative inhibition, propolis

Introduction

Propolis is the generic name for a strongly adhesive resinous substance collected by honey bees from trees and leaf buds. While propolis is produced by a variety of plants, most plant species are not known because bee collection takes place high up in trees, so it is difficult to observe (1). Only one plant species, *Baccharis dracunnculifolia*, is an established source of propolis although *Populus*, *Clusia*, and *Araucaria*, are regarded as additional source of propolis (2,3). Propolis is a resinous substance that bees collect from the exudates of plants and which they use to seal holes in the beehive (4). Propolis forms part of traditional medicine, and chemical analysis has pointed to the presence of at least 300 compounds in its composition. It is mainly composed of propolis is made of resin (50%), wax (30%), aromatic oils (10%), pollen (5%), and other organic compounds (5). The chemical composition of propolis is mainly composed of flavonoids, terpenes, amino acids, and caffeic acid phenyl esters. Several compounds have been identified in propolis are reported present that flavonoids, cinnamic acid derivatives, and terpenoids. The biological activity is basically due to the presence of phenolic compounds and flavonoids, although the exact action mechanism is unknown (6). Recently, this nontoxic natural product was reported to possess multiple pharmacological effects (7-9).

In recent years, there has been a growing interest in propolis for its biological activities, especially regarding its antioxidant, antibacterial, anticancer, antifungal, and anti-inflammatory properties (7,10,11). According to the Borrelli *et al.* (12), the antioxidant capacity of propolis is due mainly to the phenolic compounds and flavonoids, terpenes, steroids, aldehydes, and ketones. However, there are a few or no studies on the antioxidant, antimicrobial, and anticancer activities of commercial proplis have been reported. Thus in this study, we investigated the in terms of biological activities including total phenolic content, total flavonoid content, reducing power, DPPH radical scavenging effect, the scavenging activity on ABTS radical cation, antimicrobial activity, and cancer cell proliferation inhibition effect.

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Materials and Methods

Materials The propolis was provided by Seoul Propolis. Co. (Seoul, Korea). It contained 60% propolis, 39.5% water, and 0.5% glycerin. The product was aqueous and could be used to directly before analysis.

Determination of total phenol content Total phenol content was determined according to the modified a Folin-Ciocalteu method (13). A 2 mL of 2% Na_2CO_3 solution to the sample 100 μL was stirred with 100 μL of 50% Folin-Ciocalteu reagent and allowed to stand at room temperature for 3 min. The absorbance was measured with spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) at 750 nm after 30 min. The results are expressed as gallic acid (Sigma-Aldrich, St. Louis, MO, USA) equivalents, the sample total phenol content showed in mg gallic acid.

Determination of total flavonoid content Total flavonoid content was determined according to the modified method by Maria *et al.* (14). A 50 μL of sample was added to test tubes containing 100 μL of 1 M CH_3COOK, 100 μL of 10% Al $(NO_3)_3$ and 1 mL of distilled water and then stirred. The absorbance was determined spectrophotometrically at 415 nm after 40 min. Total flavonoid concentration was calculated using quercetin as standard. The total flavonoid content of the sample showed it in mg quercetin equivalent/100 g sample.

Determination of reducing power The reducing power of sample was determined according to the method of Yen and Chen (15). The concentration of sample extract was 100 mg/mL, the 250 μL of this was mixed with 250 μL of 200 mM sodium phosphate buffer (pH 6.6) and 250 μL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 250 μL of the 1% trichloroacetic acid were added, the mixture was centrifuged at 2,090×g for 10 min. The 5 mL of upper layer was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride and the absorbance was measured spectrophotometrically at 700 nm. The higher absorbance indicates higher reducing power of the samples.

Determination of DPPH radical scavenging activity Effect of propolis on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was estimated according to the method of Andreason *et al.* (16). A 50 μL of sample was added test tube containing 1 mL of 0.2 mM methanolic DPPH solution. The mixture was vortexed and left to stand at room temperature for 10 sec. A tube containing 50 μL of methanol and 1 mL of 0.2 mM methanolic DPPH solution served as the blank. The absorbance of solution was measured spectrophotometrically at 517 nm. The percentage of DPPH scavenging was obtained from the equation thus, electron donating activity (EDA, %)=$100(1-Abs_{sample}/Abs_{blank})$.

Determination of ABTS radical scavenging activity The 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation decolorization assay was modified method by Robert *et al.* (17) based on the abilities of different substances to scavenge the ABTS radical cation compared with ascorbic acid as standard antioxidant in a dose response curve. ABTS was dissolved in water to a 7.4 mM concentration. ABTS radical cation was made by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 24 hr before analysis. The concentration of ABTS radical solution was diluted with ethanol to an absorbance of 1.4 at 734 nm using the molar extinction coefficient of ABTS radical cation ($\varepsilon=3.6\times10^4$ mol/cm). A sample was added to test tube containing ABTS radical solution and left to stand at room temperature for 1 hr. The determination of sample was using the spectrophotometer at 734 nm. The results are expressed as ascorbic acid equivalents thus,

AEAC (ascorbic acid equivalent antioxidant capacity)

$$=\frac{\Delta A}{\Delta A_{aa}}\times C_{aa}\times V\times\frac{100}{W_s}$$

ΔA: change of absorbance using the sample
ΔA_{aa}: change of absorbance using the L-ascorbic acid
C_{aa}: standard concentration of L-ascorbic acid (mg/mL)
V: extract volume (mL)
W_s: weight of sample for the extract (g)

Determination of antimicrobial activity *Escherichia coli* KFRI 836, *Listeria monocytogenes* KFRI 799, *Salmonella typhimurium* KFRI 191, *Bacillus cereus* KFRI 181, *Staphylococcus aureus* KFRI 240, and *Pseudomonas aeruginosa* KFRI 252 were obtained from a Korea Food Research Institute (KFRI, Seongnam, Korea). Antimicrobial test was carried out by disc diffusion method. The microorganism were grown in a nutrient broth (Difco Laboratories, Sparks, MD, USA) and incubated at 35°C for 48 hr. A suspension of the tested microorganism (100 μL) was spread on the plate count agar (Difco, Laboratories) and the 8-mm diameter disc (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were impregnated with 2, 3, 5, 10, and 20 μL of the propolis on the inoculated plates. The inoculated plated were incubated at 37°C for 24 hr, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Cancer cell proliferation inhibition effect

***Cell proliferation*:** The test cell lines of NCI-H460 (lung cancer), MCF7 (breast cancer), MKN45 (stomach cancer), and HCT116 (colon cancer) were purchased from Korean Cell Line Bank (KCLB). The test cancer cell lines of NCI-H460, MCF7, MKN45, and HCT116 were adapted in a Rosewell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 1% of 100 unit/mL penicillin at 37°C in a 5% CO_2 incubator and used for proliferation (18).

***Anti-cancer effect by the MTT assay*:** Inhibition effect of the proliferation of the cancer cells by the sample was determined following the procedure of Young *et al.* (19). Each cancer cell such as 1.5×10^4 (NCI-H460), 2×10^4 (MCF7), 5×10^4 (MKN45), and 2.5×10^4 cell/mL (HCT116) was inoculated into a 96-well plate. After 48 hr of incubation at 37°C in a 5% CO_2 incubator, the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL) 20 μL was added and incubated for another 4 hr. The growth media was removed and dimethyl sulfoxide (DMSO) 150 μL was added into each well and dissolved. The absorbance of each well was determined by using an enzyme-linked immunosorbent assay (ELISA) microplate reader with a 550 nm (20). The growth inhibition rate was calculated as follows;

$$\text{Antiproliferative activity (\%)}=100\times\left(1-\frac{Abs_s}{Abs_c}\right)$$

Abs_s: absorbance of the sample
Abs_c: absorbance of the blank

Statistical analysis Each experiment was performed 3 times and the data are expressed as means±standard deviation (SD). Statistical analysis was performed by Duncan's multiple tests using SAS (SAS Institute, Cary, NC, USA) Software. The level of statistical significance was defined at $p<0.05$.

Results and Discussion

Total phenol and flavonoid contents In this study, the content of total phenol and flavonoid in propolis extract were 8.3 and 6.6 mg/g, respectively. According to the Jeong *et al.* (21) that the content of total phenol were 16.18, 9.31, 4.93, and 1.48 mg/g in extracted propolis by distilled water, 50% ethanol, 70% ethanol, and 95% ethanol, respectively. Jeong *et al.* (21) also reported that the main compound of polyphenol in extracted propolis by various solvent were gallic acid, chlorogenic acid, catechin, epigallocatechin gallate, epicatechin, and epicatechin gallate.

Table 1. Reducing power and DPPH radical scavenging ability of propolis

Concentration (μg/mL)	Reducing power	DPPH radical scavenging effect (%)
10	0.60±0.106[c1)]	26.28±0.416[c]
100	1.02±0.021[b]	61.30±0.700[b]
1,000	2.43±0.004[a]	82.70±0.600[a]

[1)a-c]Different letters within the same column differ significantly ($p<0.05$).

The investigated the flavonoid content of Korean propolis by Lee *et al.* (22) that total flavonoids contents of Yecheon, Youngwol, Brazilian, Chinese, and Australian propolis were 6.33, 6.43, 2.44, 6.52, and 8.11 mg/g, respectively. The geographical differences, propolis samples from Europe, South America, and Asia have different chemical compositions (23). Propolis contains many kinds of flavonoids and phenolic acid esters in Europe and China whereas; the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (24,25). Due to the differences in their chemical compositions, the biological activities of propolis from different areas are also different. In the present study, the content of total phenol and flavonoid in propolis were shown that the contents are similar to produced propolis from Korea area such as Yecheon, Youngwol.

Reducing power The result of reducing power on the propolis was shown in Table 1. The reducing power of the propolis increased with concentration of propolis ($p<0.05$). It was shown that the reducing power of 10, 100, and 1,000 μg/mL of propolis were 0.60, 1.02, 2.43, respectively. The result of high reducing power was related to result of high antioxidant activities of propolis. The similar research reported that the reducing power of the extracted propolis by various solvents increased with concentration increasing of propolis and the reducing power of propolis was 0.4 in 250 μg/mL propolis (23).

DPPH radical scavenging effect DPPH radical scavenging activity of propolis by different concentration was shown in Table. 1. It was shown at 26.28, 61.30, and 82.70% in 10, 100, and 1,000 μg/mL of the propolis, respectively ($p<0.05$). The DPPH radical scavenging activity of propolis depends on the concentration of propolis. According to the Lee *et al.* (26) reported to similar a trend that the DPPH radical scavenging activity of extracted propolis by water, 50% ethanol, 70% ethanol, and 95% ethanol were 20-30% and 65-85% in 10 μg/mL and 100 μg/mL, respectively. Kumazawa *et al.* (8) reported that antioxidant activity of propolis of various geographic origins. Chinese propolis had stronger DPPH free radical scavenging activity than propolis from Brazil was weak (8). We know that the DPPH radical scavenging activity value in the propolis was difference with various extract solvent and geographic origins. The propolis has DPPH radical scavenging activity however, more detailed qualitative and quantitative analyses of the compounds with antioxidant activity will be necessary to elucidate the antioxidant activity of propolis even though it was difficult to evaluate the quality of propolis.

Table 2. Antimicrobial activities of propolis on microorganisms

Concentration (μL/disc)	ECO[1)]	LIS	SAL	BAC	STA	PSE
2	ND[2)]	ND	ND	10.25	12.75	ND
3	9.65[3)]	9.00	9.75	11.50	13.50	ND
5	9.65	13.25	11.00	14.00	13.85	12.50
10	9.25	14.00	11.25	13.75	13.75	13.25
20	14.25	20.65	13.50	18.00	14.75	17.75

[1)]ECO, *Escherichia coli* KFRI 836; LIS, *Listeria monocytogenes* KFRI 799; SAL, *Salmonella typhimurium* KFRI 191; BAC, *Bacillus cereus* KFRI 181; STA, *Staphylococcus aureus* KFRI 240; PSE, *Pseudomonas aeruginosa* KFRI 252.
[2)]Not detected.
[3)]8.0-9.0 mm, slight low activity; 9.1-10.0 mm, middle activity; 10.1-11.0 mm, slight high activity; 11.1-12.0 mm, moderate high activity; over 12.1 mm, very high activity.

ABTS radical scavenging effect This assay was reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidant, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. Robert *et al.* (17) described that the ABTS radical scavenging assay are direct production of the blue/green ABTS radical cation chromophore through the reaction between ABTS and potassium persulfate. The ABTS radical scavenging effect of antioxidant activity on the propolis was 35.64 ±0.424 AEAC g/sample.

Antimicrobial activity Antimicrobial effect of propolis was shown in Table 2. From the concentration of 2 μL of propolis was shown antimicrobial activity against *B. cereus* and *S. aureus*. There are showing the very high antimicrobial activity against *B. cereus* and *S. aureus* from 3 μL of propolis. The microorganism such as *E. coli*, *L. monocytogenes*, and *S. typhimurium* were shown antimicrobial activity from the concentration of 3 μL of propolis. From the concentration of 5 μL of propolis was shown very strong antimicrobial activity against *L. monocytogenes* and *S. typhimurium.* There was no effect on the antimicrobial activity at 2, 3 μL of propolis however, from the concentration of 5 μL of propolis was shown very high antimicrobial activity against *P. aeruginosa*. According to the Lee *et al.* (26) the propolis extract significantly inhibited all microorganism tested, showing the largest inhibitory zone for *B. subtilis* and *B. cereus*. The results indicated that the antimicrobial effects of water and ethanol extracted propolis were greater against the growth of *S. aureus* and *Candida parapsilosis* rather than those of *Salmonella enteritidis* and *E. coli* O157:H7 (27).

Cancer cell proliferation inhibition effect Cancer cell including NCI-H460 (lung cancer), MCF7 (breast cancer), MKN45 (stomach cancer), and HCT116 (colon cancer) were used to investigate the cell proliferation inhibition effect of propolis (Fig. 1). In the results, the concentration of 100 μg/mL treated propolis was 53-73% as effective growth inhibition of all the cell lines. According to the Lee *et al.* (26) the 100 μg/mL ethanol extracted propolis exhibited the strongest inhibitory (62.63%) on A549 (lung cancer). And also, Kwon *et al.* (28) reported the 25 μg/mL propolis treated was shown 36.8% inhibition effect on the MCF7

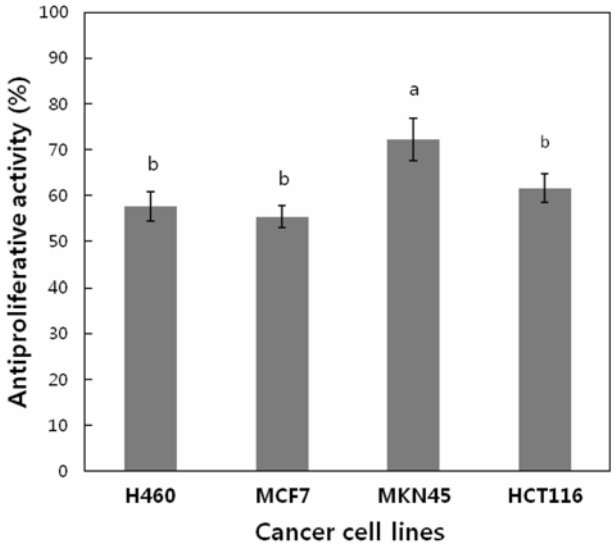

Fig. 1. Cancer cell proliferation inhibition effect of propolis.

(breast cancer).

In conclusion, the present study has demonstrated that the propolis can be used as an antioxidant, antimicrobial additive, or pharmaceutical supplement because of its beneficial biological activities such as antioxidant, antimicrobial, and cancer cell proliferation inhibition activities. The evidences of potential application of commercial propolis due to the large number of beneficial effects biological activity was shown and the propolis can be applied to the functional food, pharmaceutical, and cosmetic industry.

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