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Identification and Quantification of Propolis's Active Compound in Various Solvents

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ABSTRACT

Propolis is a resinous and adhesive substance that collected by honeybees from plant and mixed with various enzymes. The therapeutic effects of propolis such as anticancer, antibacteria, immunomodulator and antiinflammation have already known since several hundred years and had been published in many scientific journals. The therapeutic effects of propolis are sugested due to it's active compound such as CAPE, artepillin C, quercetin and hesperidine. Different extraction methods especially realted with the using of solvent influence the active component extracted from raw propolis. The aim of this study was to identify and quantify the CAPE, artepillin C, quercetin and hesperidine compound in propolis sample collected from beeskeeping in Kediri, East Java. The propolis extracted by maseration technique using different solvent such as methanol, ethyl acetate and N-heksan. Identification and quantification were performed using LC-MS/MS and LC-Quan software respectively. Various results were shown, in which the highest concentration for CAPE and quercetin were obtained from methanol. Furthermore, hesperidine and artepillin-C were reach the highest concentration in ethyl acetate and n-hexane respectively. In conclusion, methanol was the better solvent than ethyl acetat and N-heksan.

KEYWORDS: Propolis, active compound, different solvent

1. INTRODUCTION

Propolis, which also known as bee glue, is a substance produced by honeybees from resin that collected from plants and combined with wax and saliva secreted by honeybees. Propolis is used by honeybees to cover their nest wall to protect it from any outside predator entering their nest, inhibit the growth of bacteria and fungi as well [1]. Propolis is not only strengthen the cell wall, but also has an important role to protect aseptic environment inside the nest [2].

For many years, human used propolis for various diseases therapy. Propolis has already well known as a natural medicine with several functions such as antibacterial [3], antitumor [4]–[7][8], antioxidants [9], and immunomodulator [10]–[12].

Although propolis has been already used for a long time ago, but study about its chemical compounds and biological activities just have been developed in last few decades. The chemical compound of propolis is very complex, it has various chemical composition influence by type of plant as propolis source, geographic factors and also seasons [2]. The therapeutic activities of propolis have reported both as propolis whole compound and as propolis's single active compound. Some of the active chemical compounds of propolis are CAPE, artepillin C, Quercetin, and hesperidin.

CAPE is one of propolis's active compound which has benefit activities as antioxidant [13], [14], anticancer to some kind of cancer such as lung cancer [15], cervical cancer [16], breast cancer [17], and glioma [18]. Artepillin C is also an active compound of propolis which has an effective anticancer actvities to some cancer type as well, such as leukemia and colon cancer [19], [20], neurofibromatosis [21], and prostatic cancer [22]. Quercetin is also propolis's active compound which has therapeutic activities against breast cancer [23], lung cancer [24], and oral squamous cell carcinoma (OSCC) [25]. Hesperidine has anticancer activities against laryngeal cancer, cervical cancer, breast cancer [26] and also hepatocarcinoma [27].

In this study, we tried to identify several active compounds of propolis including CAPE, artepillin C, quercetin and hesperidine using different solvents such as methanol, ethyl acetate and N-heksan.

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2. MATERIALS AND METHODS

2.1 Materials

We had identified the active compound from propolis extract using four standard solutions, namely Quercetin and Artepillin C were purchased from Sigma-Aldrich. Caffeic acid phenethyl ester (CAPE) and Hesperidine were purchased from TCI. Acetonitrile HPLC grade and methanol were obtained from Merck. Water used for LC-MS/MS analysis was purified with a deionized water system. Propolis was obtained from honeybees keeping central at Pare, Kediri, East Java

2.2 Propolis Extraction

Propolis extraction process was performed based on previous method with some modification [28], [29]. Totally 50 gram propolis was extracted with 500 ml of three different solvents: methanol (SP1), ethyl acetate (SP2) and N-hexane (SP3). Macerations were conducted for 24 hours with two times shaking in the morning and evening for 30 minutes respectively. At the next day, solutions were filtered with filter paper, then the solvent was changed with the new ones and these processes were repeated till five times. After five days macerations process, solutions were incubated overnight in 4^0C, in order to separate wax from solutions. Furthermore, filtrated with whatmann filter was carried out and continued with evaporation until got the dry extracts and then the dryd extracts were kept on -20^0C.

2.3. Standard and Sample Preparation

Identification active compound was performed using Liquid Chromatography tandem Mass spectrometry (LC-MS/MS). Standard stock solutions 1000 µg/mL concentration of the individual compound were prepared separately by dissolving in methanol, except Hesperidine in methanol-DMSO (9:1) were stored at –20ºC. For calibration curve, aliquots of 1.5 to 7.0 µg/ml; 8.0 to 32 ng/mL; 10 to 42 ng/ml and 3 to 60 ng/mL were prepared from the above stocks for Quercetin, Artepillin C, CAPE and Hesperidine. Sample preparations were conducted based on previous methods with some modifications (Chorilli et al., 2011). About 0.2 – 1.0 gr the individual sample extract was weighed in centrifuge tubes with screw caps. 10 ml of the solvent methanol-DMSO (9:1, v/v) was added. Shaked vigorously for 1 minute in Branson Model 5510 ultrasonic bath for 30 minutes. The sample was centrifuged at 3000 rpm for 30 minutes, then the supernatant was transfered to centrifuge tubes with screw caps. The sample was dried in the vacuum drying oven at 90ºC, then 5 mL acetonitrile solvent was added. Sonication for 15 minutes then centrifuge at 3000 rpm for 15 minutes. Supernatant was filtered with a 0.2 μm syringe filter, and the solution was transferred to an autosampler vial and an aliquot was injected onto the LC–MS/MS system for analysis.

2.4. Apparatus and operation conditions

2.4.1. Liquid chromatography

UHPLC was performed using the Thermo Scientific (USA) Accela 1250 system. It was consisting of a quaternary pump solvent management system, an online degasser, and the autosampler was maintained at $2^{0\circ}$C. They were programmed by x-Calibur 2.1 software. Chromatographic separation was achieved using a Hypersil Gold column (50 mm × 2.1 mm,1.9 µm, from Thermo Scientific) was maintained at 40°C. A linear gradient method was compound separation in analytes. The eluent flow rate was set 300 µL/min and the elution solvents were 0.1% formic acid in water (A) and 0.1% in acetonitrile (B). The elution gradient was programmed as follows: 0.0-0.6 min., 15% B, 2.0-4.0 min 100% B, 4.1 min 15 % B and sample injection volume was 2 µL, and the run time was 5.0 min.

2.4.2. Mass spectrometry

Mass spectrometric detection was performed using Triple Quadrupole MS (TSQ Quantum Access Max, Thermo Scientific, USA). It was completed with an electrospray ionization source (ESI and the ESI source was set in negative ion mode. The HESI ionization source parameters were set as follows: Spray Voltage (3200 V); Vaporizer Temperature (350 °C); Capillary Temperature (200 °C); Sheath Gas (40 arbitrary units) and Auxiliary Gas (60 arbitrary units). The MS/MS instrument was operated in SRM (Selected Reaction Monitoring) mode with the details listed in Table 1.

Table 1. SRM MS/MS Events and Parameters

Analyte	Precusor (m/z)	Product (m/z)	Retention Time	Scan Time (min)	CE (eV)
Quercetin	301	179	2.36	1.88-2.88	15
Hesperidin	609	301	2.08	1.61-2.61	20
CAPE	283	179	2.84	2.32-3.32	20
Artipilen C	299	255	3.07	2.54-3.54	20

2.3 Quantification

Liquid chromatography-triple quadrupole tandem mass spectrometry (LC/MS/MS) enables highly selective target, sensitive confirmation and quantitation of the active compound. Chromatogram result from standards and samples were quantified by external standard (ESTD) method using LC-Quan software. Concentration of each active compound that contained in propolis extract will be known using this method. Export the quantification results to Excel and perform the calculation of individual compounds concentration.

3. RESULTS

In this study, quercetin, CAPE, hesperidin, and artepillin-C were used as a standard, in order to know the availability of each compound in propolis extract. Optimization of each standard resulted several parameter such as parent mass, product mass, retention time and collision energy (Table 1). Retention time especially was important component as a template to identify the active compounds content in propolis extract. Retention time of quercetin, hesperidin, CAPE and artepillin-C was recorded in 2.36, 2.08, 2.84, and 3.07, respectively (Figure 1).

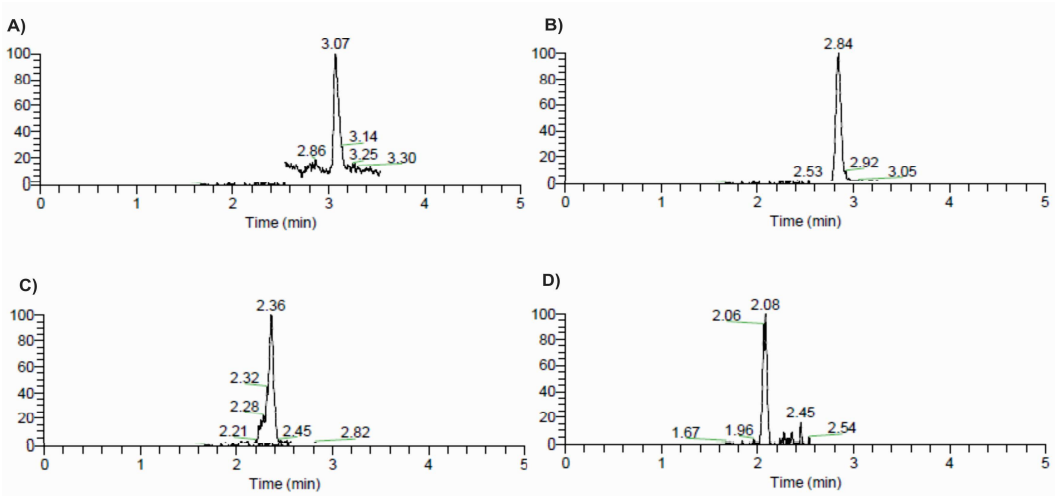

Fig. 1. LC-MS/MS results of the standard solutions

A)artepillin C with retention time 3.07; B)CAPE with retention time 2.84; C) Quercetin with retention time 2.36; D) Hesperidin with retention time 2.08.

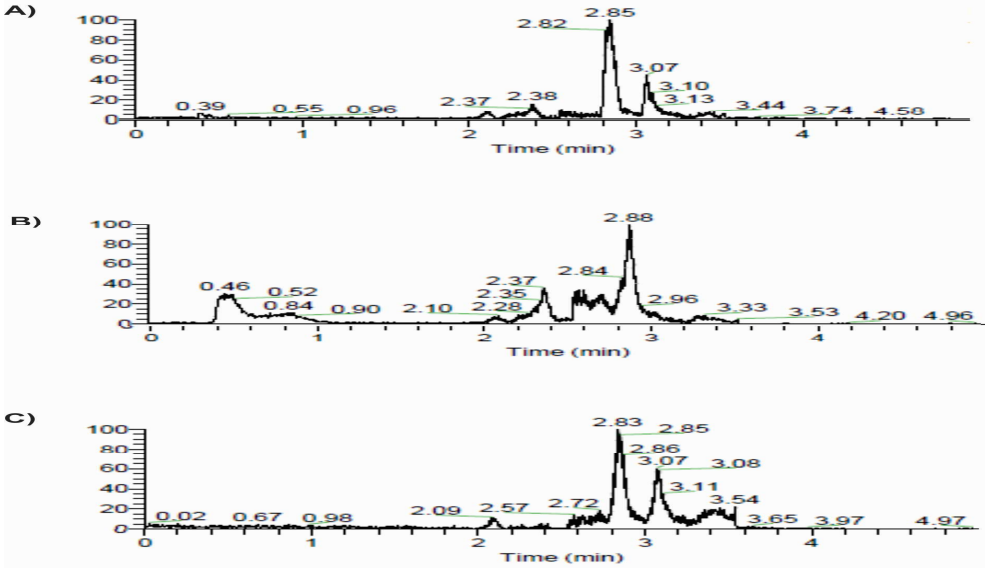

Fig. 2. LC-MS/MS results from sample of propolis extract with different

A)SP1, sample of propolis extract with methanol as a solvent; B)SP2, sample of propolis extract with ethyl acetate as a solvent; C)SP3, sample of propolis extract with N-hexane as a solvent.

Table. 2. Quantification results from LC-MS/MS for all samples

Sample	Standard Concentration (ng/gr)			
	Artepillin-C	CAPE	Quercetin ($x10^3$)	Hesperidin ($x10^3$)
SP1	82.58	2950.73	365.48	4.05
SP2	270.11	UD*	308.45	0.87
SP3	UD*	1765.42	UD*	7.63

**undetected*

Identification (Figure 2) and quantification (Table 2) analysis from sample of propolis extract with three different solvent were showed various results. The highest concentration of artepillin-C was obtained from ethyl aceate with value 270.11 ng/gr. CAPE and quercetin were concentration in methanol, 2950.73 ng/gr and $365.48x10^3$ ng/gr respectively. Furthermore, the highest concentration of hesperidine was observed in N-heksan with value $7.63x10^3$ ng/gr. Artepillin-C and quercetin were resulted a negative calculation in N-heksan because of very low concentration. Similar result also showed for CAPE in ethyl acetate. Only hesperidin was showed positive quantification in all solvents methanol (SP1), ethyl acetate (SP2) and N-hexane (SP3), although the lowest concentration was obtained in SP2.

4. DISCUSSION

Totally almost hundred compounds are identified from propolis and now still open a wide opportunity for new compounds discovery. Compounds that comprised in propolis are classified on several groups such as phenolic acids, flavonoids (flavonols, flavanones, flavanols, dihydro-flavonol), amino acids, and minerals [30]. Active compounds of propolis from various areas are vary [31]. It was affected by flora species as a source of propolis [1].

Many expertise in this field believe that propolis standardization based on its active compounds is difficult. Recently, there is no international standard related with the propolis quality because the complexity of various propolis compounds. At least, there are two factors that affected the propolis compounds. The first factor is the plant species as a source of propolis. And another factor is related to the extraction methods and solvent that used, thus possibly produce different active compounds [31].

The Different solvent will affect the compounds that extracted from propolis. In general, ethanol, methanol and water were used as a solvent in extraction methods [32]. The highest concentration of CAPE and quercetin in our study was obtained in methanol solutions. Several previous study was showed an extraction of CAPE and quercetin from propolis using methanol as a solvent [33][34]. In the previous study, artepillin C could be extracted using ethanol accompany with CAPE and quercetin [35], artepillin C that found from Brazilian propolis was optimal if extracted using ethyl acetate [20]. Similar with that study, the highest concentration of artepillin C in our study also was obtained by using ethyl acetate as a solvent. An interesting result was obtained from extraction using n-hexane as a solvent, in which the highest concentration of hesperidine was obtained. Although in several study, hesperidin could also obtained by using methanol or ethanol as a solvent [10].

In conclusion, our study prove that local propolis extract from East Java Indonesia positively contains quercetin, CAPE, hesperidin, and artepillin-C. This result also support the suggestion that various solvent will affect the concentration of active compounds that could be isolated from propolis. At least in part, it also suggested that methanol is better than other solvent since almost all active compounds result a highest concentration only artepillin C and hesperidin that resulted in second rate concentration compare to the others. Further analysis need to be conducted to examined the biological activities effect of those compounds.

Conflict of interest

The authors report no conflicts of interest.

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REFERENCES

1] K. M. Kasiotis, "Propolis non-volatile constituents : A Review," *Hygeia.J.D.Med*, vol. 6, no. 1, pp. 111–121, 2014.

[2] A. Salatino, É. W. Teixeira, G. Negri, and D. Message, "Origin and Chemical Variation of Brazilian Propolis," *eCAM*, vol. 2, no. 1, pp. 33–38, 2005.

[3] S. O. Hasson, "Antibacterial Activities Of Iraqi Propolis And Its Active Components Extracts On Some Bacterial Isolates (In Vitro Study)," *World J. Pharm. Pharm. Sci.*, vol. 3, no. 2, pp. 858–875, 2014.

[4] K. S. Borges, M. S. Brassesco, C. A. Scrideli, A. Espencer, E. Soares, and L. G. Tone, "Antiproliferative effects of Tubi-bee propolis in glioblastoma cell lines," vol. 314, pp. 310–314, 2011.

[5] S. Khacha-ananda, K. Tragoolpua, P. Chantawannakul, and Y. Tragoolpua, "Antioxidant and Anti-cancer Cell Proliferation Activity of Propolis Extracts from Two Extraction Methods," *Asian Pac J Cancer Prev*, vol. 14, no. 11, pp. 6991–6995, 2013.

[6] J. H. Naama, Z. A. Nima, and G. M. Suleiman, "Effects of Active Materials in Alcoholic Extract of Iraqi Propolis on Growth of Some Cancer Lines in The Laboratory and Cancer of Mammary Gland in Mice," bagdad, 2010.

[7] Syamsudin, P. Simanjuntak, R. Djamil, and W. L. Heffen, "Apoptosis of human Breast Cancer Cells induced by Ethylacetate Extracts of Propolis," *Am. J. Biochem. Biotechnol.*, vol. 6, no. 2, pp. 84–88, 2010.

[8] E. Szliszka, A. Mertas, Z. P. Czuba, and W. Król, "Inhibition of Inflammatory Response by Artepillin C in Activated RAW264 . 7 Macrophages," *Evidence-Base Complement. Altern. Med.*, vol. 2013, 2013.

[9] R. Padmavathi, P. Senthilnathan, D. Chodon, and D. Sakthisekaran, "Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7 , 12 dimethyl benz (a) anthracene-induced breast cancer in female Sprague Dawley rats," *Life Sci.*, vol. 78, pp. 2820–2825, 2006.

[10] S. Ansorge, D. Reinhold, and U. Lendeckel, "Propolis and Some of its Constituents Down-Regulate DNA Synthesis and Inflammatory Cytokine Production but Induce TGF- 1 Production of," *Z. Naturforchforsch*, 2003.

[11] S. Han, K. Sung, D. Yim, S. Lee, K. Cho, C. Lee, N. Ha, and K. Kim, "Activation of Murine Macrophage Cell Line RAW 264 . 7 by Korean Propolis," *Arch Pharm Res*, vol. 25, no. 6, pp. 895–902, 2002.

[12] J. M. Sforcin, "Propolis and the immune system : a review," 2001.

[13] S. Akyol, V. Ugurcu, A. Altuntas, R. Hasgul, O. Cakmak, and O. Akyol, "Caffeic Acid Phenethyl Ester as a Protective Agent against Nephrotoxicity and / or Oxidative Kidney Damage : A Detailed Systematic Review," *Sci. World J.*, 2014.

[14] U. Ozkan, A. Osun, K. Basarslan, S. Senol, I. Kaplan, and H. Alp, "Effects of intralipid and caffeic acid phenethyl ester on neurotoxicity , oxidative stress , and acetylcholinesterase activity in acute chlorpyriphos intoxication," *Int J Cin Exp Med*, vol. 7, no. 4, pp. 837–846, 2014.

[15] M. Chen, C. Wu, Y. Chen, P. C. Keng, and W. Chen, "Cell Killing and Radiosensitization by Caffeic Acid Phenethyl Ester (CAPE) in Lung Cancer Cells," vol. 45, no. 2, pp. 253–260, 2004.

[16] T. Hsu, C. Chu, M. Hung, H. Lee, and H. Hsu, "Caffeic acid phenethyl ester induces E2F-1-mediated growth inhibition and cell-cycle arrest in human cervical cancer cells," *FEBS J.*, vol. 280, pp. 2581–2593, 2013.

[17] S. S. Ulasli, S. Celik, E. Gunay, M. Ozdemir, A. Ozyurek, T. Koyuncu, and M. Unlu, "Anticancer Effects of Thymoquinone , Caffeic Acid Phenethyl Ester and Resveratrol on A549 Non-small Cell Lung Cancer Cells Exposed to Benzo (a) pyrene," *Asian Pac J Cancer Prev*, vol. 14, pp. 6159–6164, 2013.

[18] T. Tseng, C. Shen, W. Huang, C. Chen, W. Liang, and T. Lin, "Activation of neutral-sphingomyelinase , MAPKs , and p75 NTR-mediating caffeic acid phenethyl ester – induced apoptosis in C6 glioma cells," *J. Biomed. Sci.*, vol. 21, no. 1, pp. 1–11, 2014.

[19] Y. Akao, H. Maruyama, Matsumoto, I. Kaenji, K. N, T. S. Akamoto, Y. A. Raki, S. M. Ishima, and Y. N. Ozawa, "Cell Growth Inhibitory Effect of Cinnamic Acid Derivatives from Propolis on Human Tumor Cell Lines," vol. 26, no. July, pp. 1057–1059, 2003.

[20] K. Shimizu, S. K. Das, T. Hashimoto, Y. Sowa, T. Yoshida, T. Sakai, Y. Matsuura, and K. Kanazawa, "Artepillin C in Brazilian propolis induces G0/G1 arrest via stimulation of Cip1/p21 expression in human colon cancer cells," *Mol. Carcinog.*, vol. 44, no. 4, pp. 293–299, 2005.

[21] S. M. Messerli, M. Ahn, K. Kunimasa, M. Yanagihara, T. Tatefuji, K. Hashimoto, V. Mautner, Y. Uto, H. Hori, S. Kumazawa, K. Kaji, T. Ohta, and H. Maruta, "Artepillin C (ARC) in Brazilian Green Propolis Selectively Blocks Oncogenic PAK1 Signaling and Suppresses the Growth of NF Tumors in Mice," *Phytother.Res*, no. June, 2008.

[22] E. Szliszka, Z. P. Czuba, M. Domino, B. Mazur, G. Zydowicz, and W. Krol, "Ethanolic Extract of Propolis (EEP) Enhances the Apoptosis- Inducing Potential of TRAIL in Cancer Cells," pp. 738–754, 2009.

[23] X. Deng, H. Song, Y. Zhou, G. Yuan, and F. Zheng, "Effects of quercetin on the proliferation of breast cancer cells and expression of survivin in vitro," *Exp. Ther. Med.*, vol. 6, pp. 1155–1158, 2013.

[24] S.-Y. Zheng, Y. Li, D. Jiang, J. Zhao, and J.-F. Ge, "Anticancer effect and apoptosis induction by quercetin in the human lung cancer cell line A-549.," *Mol. Med. Rep.*, vol. 5, no. 3, pp. 822–6, 2012.

[25] S. Chen, S. Nien, C. Wu, C. Liu, and Y. Chang, "Reappraisal of the anticancer efficacy of quercetin in oral cancer cells," *J. Chinese Med. Assoc.*, vol. 76, no. 3, pp. 146–152, 2013.

[26] Al-Ashal and El-Sheltawy, "Antioxidant capacity of hesperidin from citrus pel using electron spin resonance and cytotoxic activity against human carcinoma cel lines.," *Pharm Biol*, vol. 49, no. 3, pp. 276–82, 2011.

[27] S. A. Fathy, F. Abde-Hamid, S. Agwa, and S. El-Diasty, "Provided for non-commercial research and education use . Not for reproduction , distribution or commercial use . The Anti-Proliferative Effect of Hesperidin on Hepatocarcinoma Cells HepG2," *Egypt. Acad. J. Biol. Sci.*, vol. 6, no. 2, 2014.

[28] N. Orsolic, S. Terzic, Z. Mihaljevic, S. Lidija, and I. Basic, "Effects of Local Administration of Propolis and Its Polyphenolic Compounds on Tumor Formation and Growth," *Biol Pharm Bull*, vol. 28, no. 10, pp. 1928–1933, 2005.

[29] L. Paviani, P. Sacoda, E. Saito, and F. Cabral, "Extraction techniques of red and green propolis : extraction yield of phenolic compounds."

[30] Marghitas, D. Dezmirean, A. Moise, C. M. Mihai, and L. S. Laslo, "DPPH Method for Evaluation of Propolis Antioxidant Activity," *Bull. UASVM Anim. Sci. Biotechnol.*, vol. 66, pp. 253–258, 2009.

[31] V. Bankova, "Chemical diversity of propolis and the problem of standardization," *J. Ethnopharmacol.*, vol. 100, pp. 114–117, 2005.

[32] I. B. S. Cunha, A. C. H. F. Sawaya, F. M. Caetano, M. T. Shimizu, M. C. Marcucci, F. T. Drezza, G. S. Povia, and P. D. O. Carvalho, "Factors that Influence the Yield and Composition of Brazilian Propolis Extracts," vol. 15, no. 6, pp. 964–970, 2004.

[33] M. J. Valente, A. F. Baltazar, R. Henrique, L. Estevinho, and M. Carvalho, "Biological activities of Portuguese propolis : Protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth in vitro," *Food Chem. Toxicol.*, vol. 49, no. 1, pp. 86–92, 2011.

[34] L. Barrientos, C. L. Herrera, G. Montenegro, X. Ortega, J. Veloz, M. Alvear, A. Cuevas, N. Saavedra, and L. A. Salazar, "Chemical and botanical characterization of chilean propolis and biological activity on cariogenic bacteria Streptococcus mutans and Streptococcus sobrinus," *Brazilian J. Microbiol.*, vol. 44, no. 2, pp. 577–585, 2013.

[35] M. Jang, S. Sheu, C. Wang, Y. Yeh, and K. Sung, "Optimization Analysis of the Experimental Parameters on the Extraction Process of," in *International Multi Conference of Engineers and Computer Scientists*, 2009, vol. II, pp. 18–22.