GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
(**Published By**: Global Institute for Research & Education)
www.gifre.org

Analysis of Active Components of *Trigona* spp Propolis from Pandeglang Indonesia

Akhmad Endang Zainal Hasan[1], I Made Artika[1], Kuswandi[2], & Gerardus Diri Tukan[3]

[1]Department of Biochemistry, Faculty of Mathematics and Natural Sciences,
Bogor Agricultural University, Indonesia
[2]Husbandary Indonesian Institute of Sciences (Balitnak), Bogor, West Java, Indonesia
[3]Department of Chemistry Education, Faculty of Education and Educative Sciences,
Widya Mandira Chatolic University (UNWIRA), Kupang, Indonesia

Abstract

Propolis has been shown to have anticancer, antifungi and antimicrobial activity. This research was aimed to analyze the active compounds of propolis through fractination using the Thin Layer Chromatography (TLC) and column chromatography methods followed by determination of the antibacterial activity of each faction. The active agents from the fraction showing highest antibacterial activity were then determined by GC-MS technique. Resulted showed that the active agents were present in eight different fractions. Test on antibacterial activity against *E. coli* showed a fraction (denoted c fraction) has the highest antibacterial activity. GC-MS analysis showed that c fraction contained at least 24 compounds. The most abundance compound from c fraction was similar to 9, 19-cyclolanost-24-en-3-ol, (3.beta)-(CAS) or cycloartenol. This compound had retention time of 40.25 minute and area of 49.91% out of the total area.

Keyword: propolis, *Trigona* spp, antibacterial agent, active compounds.

Introduction

Study on propolis of *Apis melifera* has revealed compounds of propolis fractions from different locations (Teixeira et al., 2005). Yaghoubi *et al.* (2007) reported that propolis from Iran contains pinochembrin, caffeic acid, caemferol, phenethyl caffeate, chrysin, and galangin. Total flavonoid and phenolic was 7,3% and 36% repectively, both of which strongly inhibit microbial growth. Kosalec et al. (2004) reported that raw propolis contains 50% resin (fraction polyphenolic fraction), 30% wax, 10% essential oil, 5% pollen and 5% organic compound and mineral. The chemical composition of propolis is very complex and it contains more than 200 types of compound. Propolis also contains flavonoids that are very high, so that many researchers who align with propolis flavonoids (Chang et al., 2008). Khismatullina (2005) revealed that propolis with a number of compounds display a variety of biological effects and pharmacological activity.

Trusheva et al. (2006) was analyzed the active components of red propolis from Brazil using column chromatography technique and Nuclear Magnetic Resonance (NMR) spectroscopy. They discovered at least 14 different compounds the which includes simple phenols, triterpenoids, isoflavonoids, prenilated benzophenon and naptoquinon epoxide (compounds isolated from nature). Three of the components were reported to have strong antibacterial and antifungal activity observed using petri disc that method and two compounds have the ability to scavanger free radical based on antioxidant test using 1,1- diphenyl-2-picrylhhydrazyl (DPPH) method. According Bankova et al. (2000), physical properties and chemical composition of propolis and propolis properties are very dependent on where the bees obtain botanical resin, as well as season and geographical conditions of the region or place where propolis is found. In temperate regions such as Europe, Asia and North America, propolis obtained from this area have a similar chemical composition to the main phenolic material: aglycone flavonoids, aromatic acids and esters. Propolis from the tropics region, particularly Brazil, showed some chemical components and biological activity. Chemicals that act as antibacterial which are prenylated p-coumaric acids**:** 3,5-diprenyl-4-hydroxycinnamic acid, 3-prenyl-4-dihydrocinnamoy-loxycinnamic acid, and 2,2-dimethyl-6-carboxy-ethenyl-2H-1-benzopyran. Lignans : 3-acetoxymethyl-5-[(E)-2-formylethen-1-yl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran, sesamin, achantin, and sesartenin. Diterpenic acids : 15-oxo-3,13Z-kolavadiene-17-oic acid and its E-isomer, communic acid, imbricatoloic acid, and isocupressic acid. While the act as cytotoxic and immunomodulating are flavonoid **:**aromadendrine-4'methyl ether and 3,5,7-trihydroxy-6,4'-dimethoxyflavon; prenylated p-coumaric acids: 3,5-diprenyl-4-hydroxynnamic acid and 9-E-,2-dimethyl-6-carboxyethenyl-8- prenyl-2H-1-benzopyran; Lignans : 1-(4-hydroxy-3-metho phenyl)-2-{4-[(E)-3-acetoxy- propen-1-yl]-2-ethoxyphenoxy}propan-1,3-diol 3-acetate (eryth ro and treo) and Yangambin. Diterpenic acid : ent-17-hydroxy-3,13Z-clerodadien-15-oic acid. Caffeoylquinic acids **:** 3-caffeoylquinic (chlorogenic) acid, 4-caffeoylquinic acid, 5-caffeolyquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid methyl ester. Based on the plant origin of propolis resin forming the active compounds as well as knowledge of propolis, Bankova et al. (2000) found that plants of *Populus* spp from Europe, Asia and North America can produce propolis with components pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, galangin, caffeates (benzyl, phenylethyl, prenyl), while the plant *Betula verrucosa* (birch) from North Russia produce acacetin, apigenin, ermanin, rhamnocitrin, kaemferid, α-acetoxybetulenol. Propolis from *Baccharis* spp and *Araucaria* spp containing prenylated p-coumaric acids, prenylated acetophenones, and diterpenic acids

Method

Crude Propolis Sample. Trigona spp honey comb taken at several locations bee keeping in Pandeglang, Banten Indonesia on July.

Extraction Propolis Trigona spp. Extraction of propolis from the beehive Trigona spp done by the method of Hasan et al. (2007) and Hasan et al. (2011). The 150 g bee hive, macerated with 650 ml of ethanol 70% (soaked while shaken using a shaker) for 7 days in the Erlenmeyer 1000 ml. The filtrate obtained, united in a dark container, then freeze-dried to form the solid extract. Then these are used for subsequent testing.

Antibacterial Activity Test and Determination of Minimum Inhibitory Concentration Growing (MIC). Testing was conducted using the antibacterial activity of perforation or diffusion wells (Andrews, 2001). Samples for testing antibacterial activity of propolis is a solution made six series of concentration. For the positive control used solution ampicillin 100 ppm, whereas the negative control used solvent propylene glycol and ethanol 70%. Also used propolis X (commercial) for comparison.

MIC test (well diffusion method). A total of 50 µl of bacteria results inoculation (106 cfu/ml in liquid media put into a sterile petri dish and then added with 10 ml of sterile media to 50^{o}C (which is not yet solidified). Mixture was homogenized in a petri dish by means of a petri dish slowly shake-land forming a figure eight on the surface of the laminar table. Mixture was allowed to stand until solidified and then made holes (wells) in diameter about 5 mm by using a pipette. Then added to each well in a 50 mL solution of propolis extract for each concentration and also the comparison with the control (positive and negative) on the other wells. Done triplo. Cup sealed and incubated at 37 °C for 24 hours. After 24 hours of incubation, observed and measured in the clear area around the wells that showed growth inhibition ability of bacteria (antibacterial activity).

Search In Active Compounds in Propolis (TLC method). Identification of compound fractions. Analysis of the fraction of compounds in propolis samples Trigona sppdilakukan by thin-layer chromatography (TLC) and preparative TLC. TLC plates silica gel G60 F254 as stationary phase while the mobile phase is a solvent mixture of n-hexane:chloroform:ethanol 90% with a ratio of 2:1:0.1 (v/v). The types of solvents used as an eluent mixture, identified in advance by a single eluent test. TLC results were observed with a 254 nm UV lamp.

Fractionation. Separation and purification of each fraction (fractionation) performed by column chromatography. Chromatography column filled with colloidal silica gel GF254. Solvent used, either to dissolve propolis extract samples, the manufacture of colloidal silica gel G60 F254 for filling the column, and the eluent, the solvent is a mixture of n-hexane:chloroform:ethanol 90%, 2:1:0.1 (v/v).

A total of 2 g of propolis extract was diluted with 5 ml of solvent mixture (eluent), then put on the top surface of the column. Slowly, the eluent is added from the top surface of the column, while the fractions collected every 5 ml at the lower end of the column with a flow rate of 2 ml/min. Fractions obtained solution, and then analyzed by TLC plates and observed with a 254 nm UV lamp. The same spots, grouped to produce a fraction groups.

Fraction activity test compounds against bacterial activity. Each group fraction from the fractionation of compounds tested antibacterial activity against *E.coli*, with the well diffusion method.

Identification of Active Compounds faction. The group has antibacterial activity fraction was isolated and identified the largest of its kind by the method of gas chromatography-mass spectrometry (GC-MS). GC-MS instrument used Agilen Technologies 6890 Gas Chromatograph with auto sample and mass selective detector 5973 and Chemstation data system. The column used was a capillary column HP Ultra 2, (17 mm x 0.25 mm), and diameter of 0.25 ml. A total of 5 ml sample (c fraction) is injected at a temperature of 250 ^{o}C. Carrier gas was helium, with a constant flow rate system, at 0.9 mL/min.

Results and Discussion

The test results of the antibacterial activity of the fractions and controls, are shown in Table 1. Treatment of 6 times, it appears that all groups antibacterial effect faction, which inhibit the growth of *E. coli*, which is indicated by the size of the diameter of the clear zone is formed. Clear zone are listed in Table 1 seem less clear, this is caused by a number of factors that lower test bacterial population, giving rise to a thin clear zone. The lower of the population of test bacteria are put into the cup petri dish, can be caused by pipetting in homogeneous against bacterial culture results in liquid media, as well as the biomass of bacteria culturing in liquid media result not the maximum.

Based on the test results of the antibacterial activity of the fractions of the bacterium E. coli, it is known that the fraction of C has the greatest antibacterial activity (Figure 1). Based on the test results of the antibacterial fraction, the c fraction is used to further test the active component searches through GC-MS technique. Although c fraction had a higher inhibitory power, but did not give a fraction between the inhibitory effect was significantly different (P> 0.05).

The results of GC-MS analysis of the c fraction (fraction which has the highest antibacterial activity), are known to peak at 24 percent retention times and different areas (Figure 1). Many as 10 percent of them have a peak in the area below 1%, which indicates the low abundance components of the compound in the sample C fraction. While 14 percent of the area has other peak above 1%, which indicates a relatively high abundance in the sample c fraction. the review and further discussion is on the 14th peaks.

Figure 1. GCMS Propolis C Fraction

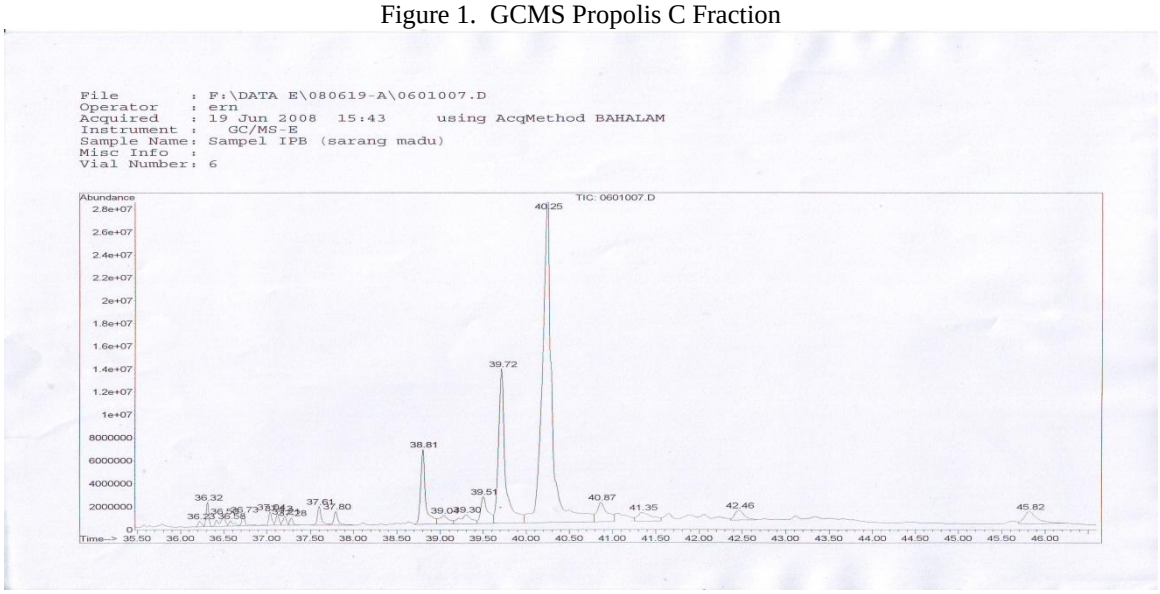

Table 1. The test results of the antibacterial activity of propolis fractions Trigona spp Pandeglang origin of the *E. coli*

No	Fraction and control	Area zona plaque (mm)/reply to.....						Averages
		1	2	3	4	5	6	(mm)
1	Fraction A	3,00	4,79	3,00	5,95	3,55	4,65	4,156)^{ab}
2	Fraction B	3,15	3,35	5,25	4,00	4,35	5,25	3,792)^{ab}
3	C fraction	2,65	4,85	6,00	5,95	4,55	2,65	4,442)^{ab}
4	Fraction D	4,25	4,00	5,15	4,00	3,85	2,55	3,967)^{ab}
5	Fraction E	3,95	3,25	4,25	4,15	2,85	3,35	3,633)^{ab}
6	Fraction F	2,30	2,25	3,15	5,25	2,25	3,75	3,158)^{a}
7	Fraction G	1,75	3,85	3,75	4,45	3,45	3,25	3,417)^{ab}
8	Fraction Non Soluble	2,75	4,35	3,75	3,55	3,25	3,75	3,567)^{ab}
9	Propolis Crude Extract	3,45	3,65	5,65	4,25	4,35	3,75	4,184)^{ab}
10	Solvent PG + aquadest (1:2)	2,75	2,25	2,55	3,45	2,65	2,25	2,650)^{a}
11	Ampicilin 1000 ppm	3,25	5,85	5,25	6,00	5,35	5,25	5,158)^{b}

Remark: Result (supercript) followed by the same superscript letter on the same row shows that the results were not significant at α=0.05.

Retention time of the 14th peaks is different and has a range of 28.38 to 45.82 minutes indicate a difference in the characteristics of the compound. This is evident from the graphic display of molecular mass fragments of each peak. The results of this analysis can reveal the types of compounds contained in fractions C, although still a comparative approach to the structure of the data bank. The similarity of the structure of the compound component is expressed by the percent equilibrium, whereas the abundance of the compound component is expressed as a percent of the area. Summary of percent similarity structure of compound C fractions can be seen in Table 2.

In Table 2, the 2nd peak with a retention time of 30.33 minutes (Figure 1), there is a compound which has a 89% similarity with ethyl linoleic or linolenic acid, with an abundance of 2.06%. Linoleic acid is an omega-3 fatty acids, because the C=C bond first started on the third carbon atom from the CH_3 end. Because dealing with fats and oils, it is also known as linoleic acid fatty acids. Linoleic acid, including esters such naturally occurring fats and oils of animal or vegetable (Soetrisno et al., 2003).

Linoleic acid and linolenic acid are two types of essential fatty acids, which must be administered through food because it can not be synthesized in the body, while the body is in dire need. Today formulated in the form of linoleic acid conjugated (conjugated linoleic acid /CLA) because of its potential to improve the health of individuals who consume them. Preparations is through milk, dairy products such as yogurt and cheese, and meat. In a variety of research, both in experimental animals, and human cell cultures, it is known that these nutrients may prevent the accumulation of fat, are antioxidants, which can fight free radical damage, inhibit the growth of cancer, heart disease, diabetes and obesity, as well as to stimulate immune function and growth factors (Hidayat, 2006).

Linoleic acid content in propolis *Trigona* spp Pandeglang origin suggests that bees *Trigona* spp which produce propolis resin consumption of coconut trees around it. Because the plant is a plant oil. producing unsaturated fatty acid. and linoleic acid with an average abundance 2.70% (Soetrisno, 2013).

Table 2. Summary percent estimate structural similarity of the compounds of C fraction

Peak	Retention time (min)	Area (%)	Structure Analog	Similarity (%)
1	28.37	1.56	*1-(2.6.6-trimetil-1-cyclohexen-1-yl)-3-methyl-2-heptene*	38
2	30.33	2.06	*Ethyl linoleate*	89
5	36.32	1.05	*2.4-di-t-butyl-1.5.6.7-tetraisopropil2.4-diaza-3-deoro-closo-heptaborane*	72
13	37.61	1.17	*Alpha-ethyl-ortho-metoxybenzil alkohol*	27
15	38.81	5.80	*Lanosta-8.24-dien-3-ol. (3.beta.)-(CAS)*	95
16	39.04	1.31	*Obtusifoliol Ergosta-8.24 (28)-dien-3-ol. 4.14-dimethyl-. (3.beta..4.alpha.. 5.alpha.) -(CAS)*	53
17	39.30	1.77	*Lanosta-8.24-dien-3-ol.(3.beta)-(CAS)*	99
18	39.51	3.14	*Lanosta-8.24-dien-3-ol.(3.beta)-(CAS)*	98
19	39.72	16.97	*Viminalol Urs-12-en-3-ol. (3.beta.)-(CAS)*	86
20	40.25	49.91	*9.19-cyclolanost-24-en-3-ol. (3.beta.)-(CAS)*	99
21	40.87	3.18	*9.19-cyclolanostan-3-ol.24-methylene-. (3.beta.)-(CAS)*	99
22	41.35	2.02	*12.13-Dimethoxytotara-8.11.13-triene phenanthrene. 1.2.3.4.4a.9.10a-octahydro-6.7-dimethoxy-1.1.4a-trimethyl-8-(1-methylethyl)*	58
23	42.46	1.49	*Dammara-20.24-dien-3-ol*	70
24	45.82	2.57	*Ethyl vallesiachotamate*	46

In nature. there is also a linoleic acid in rice bran. This causes the rice bran is known to have high nutritional value. The nutritional value of rice bran this occurred because the content of linoleic acid. which has the biological activity of nature. and with the other contained components such as oryzanol. tocopherols. tocotrienols. phytosterols. polyphenols and squalene induce antioxidant properties (Goffman and Bergman, 2004).

At the peak to 15. 17 and 18. c fraction containing compound has a great opportunity Lanosta-8. 24-dien-3-ol.(3beta). At the peak to 15. the retention time of 38.81 minutes and 5.80 percent area. Peak to 17 had a retention time of 39.30. with an area of 1.77 percent. While the peak to 18 . has a retention time of 39.51 with 3.14 percent area. Of the third peak. each one percent similarity structure of compounds with compounds present in the c fraction were 95 % . 99 % and 98 %. This suggests that there is a component c fraction compounds has a great chance to have the same structure as Lanosta-8.24-dien-3-ol.(3 beta). Compounds Lanosta-8.24-dien-3-ol.(3 beta) is equivalent to the 4.4.14 alpha-trimethyl-5 alpha-cholesta-8 .24-dien-3 beta-ol–(CAS); also called cryptosterol-CAS of family lanosterol. a precursor compound for cholesterol metabolism and cucurbitacins (Aoyama et al., 1983)

At the peak to 19. there is a component compounds in c fraction that has a great chance it contains a compound similar to compound viminalol (a-amyrine;12-Ursen-3-ol). The existence of these compounds in c fraction. appears with a retention time of 39.72 minutes. the percent area of 16.97 %. Opportunities similarity component of this compound is 86 percent. Viminalol compound is a compound that has a biological activity. as antineoplastic compounds. the antibiotic used as an anticancer compound . Viminalol also known as a potent anti-HIV (Otuki et al., 2005).

Another type of compound that has the highest abundance in the c fraction is the similarity with the structure of compound 9.19-cyclolanost-24-en-3-ol. (3.beta.) - (CAS). or Cycloartenol. This compound appears through the peak to 20. with a retention time of 40.25 minutes. and the percent area of 49.91%. Opportunities structural similarity of these compounds with compounds present in c fraction are 99%. Based on the percent of the area. it can be said that in the C fraction of propolis Trigona spp. dominated by similar compounds 9.19-cyclolanost-24-en-3-ol.(3.beta.)-(CAS). or Cycloartenol. Compound 9.19-cyclolanost-24-en-3-ol. (3.beta.)-(CAS). or Cycloartenol a steroid precursor formation in plant tissue. These compounds. together with lanosterol. is formed from the conversion of acetic acid through a mevalonic acid and squalene (a terpenoid). in a series of steroid biosynthesis. The structure resembles a triterpenoid lanosterol (Lenny, 2006).

Cycloartenol is a component of making K-liquid chlorophyll. which is a health beverage preparation believed to help detoxify and reduce toxins in the body. balancing the system hormoral and acid-base balance in the body. Drink supplements that contain efficacious cycloartenol also increase nutrient intake in the blood to increase oxygen in the blood. helping the regeneration of red blood cells. Inhibit the oxidation process and stimulates cell regeneration. as well as being inhibitors of bacterial growth (Matsuda et al., 2000). In addition to containing cyclolanost. c fraction Trigona spp propolis also contains other compounds that are similar to the 9.19-cyclolanostan-3-ol.24-methylene-3.beta. which has a retention time of 40.87 minutes. 3.18 percent area %. and the chance of structural similarity of 99%. Compound 9.19-cyclolanostan-3-ol.24-methylene-3.beta. as an anti-HIV compound used to prevent the HIV virus (Verotta et al., 1998).

There are also compounds in propolis *Trigona* spp C fraction which has similarities with Dammaradienol or Dammara-20.24-die(3S.8R.10R.14R)-4.4.8.10.14-Pentamethyl-17-(5-methyl-1-methylene-hex-4-enyl)-hexadecahydro-cyclopenta[a]phenanthren-3-ol.) which has a retention time of 42.46 minutes. an area of 1.49%. and 70% chance of similarity with the structure of the compound is in the C fraction of propolis Trigona spp. Dammara is a resin from the plant. and is used as a varnish on the wood and paint making materials (Doelen and Boon. 1998) as well as desinfectant. Based on the percent area (Table 8). which revealed an abundance of types of compounds contained in the C fraction of

propolis Trigona spp Pandeglang origin. it appears that the compound cyclolanost-9.19-24-en-3-ol. (3.beta.) - (CAS) or Cycloartenol is the component with the greatest abundance. and the similarity of 99%.

Compound type *9.19-cyclolanost-24-en-3-ol. (3.beta.)-(CAS)* or Cycloartenol. and compound *viminalol (a-amyrine; 12-Ursen-3-ol)* obtained as two of the components in the C fraction of propolis Trigona spp Pandeglang origin. has in common with the search results by Trusheva et al. (2006). the active compounds in Brazilian red propolis . It was found that compounds of this type are abundant in the Brazilian red propolis. and is the type of alcohol triterpenic. These compounds are shown to have antibacterial activity. antimycotic and anti-free radical. Antibacterial activity test conducted on bacterial *Stapylococus aureus. E. coli* and *Candida albicans*. it is known that these compounds at a concentration of 0.4 mg / 0.1 ml of ethanol. strongly inhibits the growth of three types of bacteria. It was also found that the color red and the efficacy of Brazilian propolis is similar to the typical red propolis origin Cuba and Venezuela. Where. plant sources identified in Cuba in the surrounding area boast Cuban red propolis. is *Clusia nemorosa* (Clusiaceae). While in Venezuela. bees collect from plants *Clusia scrobiculafa*.

Several reference compounds that have similar structures with some compounds in the C fraction of propolis Trigona sp Pandeglang Indonesian origin is 1. Ethyl linoleat. **2.** Lanosta-8.24-dien-3-ol.. **3.** 12-Ursen-3-beta-ol; viminalol; alpha-amyrenol; alpha-amyrine.. **4.** 9-beta.19-cyclo-24-lanosten-3beta-ol atau (3beta)-9.19-Cyclolanost-24-en-3-ol.. **5.** (3beta.9xi.10xi)-24-Methylene-9.19-cyclolanostan-3-ol.. **6.** Dammara-20.24-dien-3-ol.

Conclusion

Fractionation Trigona spp propolis Pandeglang Indonesia origin by column chromatography techniques. produces 8th groups of fractions (fraction groups A to H). Group C fraction has the greatest antibacterial activity against *E. coli*. Based on the retention time. c fraction contains 24 kinds of compounds. From this analysis it is known that in the C c fractionontained compounds that have the highest abundance (49.91%). which is similar to the compound 9.19-cyclolanost-24-en-3-ol. (3.beta.)-(CAS) atau Cycloartenol with 99% similarity.

Acknowledgement

The authors wish to thank to BPPS Fellowship of Indonesian Ministry of Education and Culture and Bogor Agricultural University for its facilities for conducting this study.

Reference

Andrews, J.M. 2001. Determination of minimum inhibitory concentrations. J Antimicrobial Chem. 48(1); pp.5-16

Aoyama, D,B.Y., Yoshida Y., Katsuki, U.S.M., Mohan V.P., Hata S., Nishino T.H., Sprinson D.B. 1983. Altered cytochrome P-450 in a yeast mutant blocked in demethylating C-32 of lanosterol. *J. Biol. Chem*. 258, pp.9040-9042.

Bankova V.S., de Castro S.L., Marucci M.C. 2000. Propolis : Recent advances in chemistry and plant origin. Apidol, 31, pp.3-15.

Chang R, D Piló-Veloso, Sal M., Nascimento E.A. 2008. Analysis of a Brazilian green propolis from *Baccharis dracunculifolia* by HPLC-APCI-MS and GC-MS. *Rev Bras Farmacog*. 18:pp.549-556.

Goffman, F.D. and Bergman C.J. 2004. Rice Kernel Phenolic Content and Its Relationship with Antiradical Efficiency. J Sci Food Agr, 84:pp.1235-1240.

Hasan A. E. Z., I. M. Artika, A. Fatoni, Kuswandi, and B. Haryanto. 2011. Antibacterial activity of propolis *Trigona* spp from Bukittinggi, West Sumatera against *Salmonella* sp. Chem. Progress. 4(2):pp.55-59.

Hasan A. E. Z., I. M. Artika, Kasno, and A. D. Anggraini. 2007. Antibacterial activity test of *Trigona* spp propolis. *In*: Arifin B, T Wukirsari, S Gunawan, WT Wahyuni. National Seminar of Indonesian Chemistry Association. Bogor, September 12, 2006. Department of Chemistry, IPB and Indonesian Chemistry Association. pp. 204-215.

Hidayat, B. 2006. Fortification of DHA and AA to the baby, the role and benefits. http://www.pediatrik.com. [12 July 2013].

Khismatullina N. 2005. *Apitherapy : Guidelines for more effective use.* Mobile Ltd. Rusia.

Kosalec, I., Bakmaz M, Vladimir-Knezevic S, Pepeljnjak S. 2004. Quantitative analysis of flavonoids in propolis samples from Northern Croatia. *Acta Pharm*. 54, pp.65–72.

Lenny S. 2006. Compounds of Terpenoids and Steroids. FMIPA, University of Northern Sumatera, Medan Indonesia.

Matsuda S.P., Darr L.B., Hart E.A., Herrera J.B., McCann K.E., Meyer M.M., Pang J., Schepmann H.G. 2000. Steric bulk at cycloartenol synthase position 481 influences cyclization and deprotonation. Org Lett. 2(15):pp. 2261-2263.

Otuki, M.F. Ferreire J., Lima F.V., Meyre-Silva C., Malheiros A., Muller L.A., Cani G.S, Santos A.R., Yunes R.A., Calixto J.B. 2005. Antinociceptive properies of a mixture of α-amyrin and β-amyrin triterpenes: evidence for participation of Protein Kinase C and Protein Kinase A pathways. *J. Pharmacol. Exp. Ther*. 313(1):pp.310-318.

Soetrisno. 2013. Introduction to Esther. http:// www.chem-is-try.org. [18 July 2013]

Teixeira E.W., G. Negri, R.M.S.A. Meira, D. Message, and A. Salatino. 2005. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. Evid Complement Alternat Med. 2(1):pp.85–92.

Trusheva B., Popova L., Bankova V., Simova S., Marcucci M.C., Miorin P.L., Pasin F.R., Tsvetkova I. 2006. Bioactive constituents of Brazilian red propolis. *eCAM*., 3(2), pp. 249-254

Verotta L. Tato M. El Sebakhy NA. Taoima SM. 1998. Cycloartane triterpene glycosides from *Astragalus sieberi*., Phytochemistry-Oxford, Oxford, Elsevier Science Ltd, 48(8). pp.1403-1409

Yaghoubi, S.M.J., Ghorbani G.R., Soleimanian Z.S., Satari R. 2007. Antimicrobial activity of Iranian propolis and its chemical composition. *DARU*. 15(1):pp.45-48.