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Development of microwave energy to the fast extraction of policosanol from beeswax

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Abstract—**Policosanol is a mixture of long chain aliphatic primary alcohols (C20 to C36). It has been use in pharmaceutical composition and food supplements. The main objective of this study is developing policosanol extraction from beeswax by microwave energy activation. The beeswax contained 0.2-0.5 % of moisture and melted at 58-62℃. Triglycerides and other impurities were extracted from crude beeswax by refluxing with hexane followed by isopropanol. The purified beeswax was complete rapidity hydrolysis with 0.5 M KOH in 80% ethanol in 60 seconds at 300 watt of microwave instrument in activation. Long chain aliphatic alcohols (policosanol) and free fatty acids of the hydrolyzed product were displayed by TLC. Purification of policosanol was performed by extracting with a mixture of organic solvent and the good policosanol yields microwave were 14.57%. The results of policosanol composition from gas chromatography showed that policosanol consisted of 18-34 carbon atoms of long chain aliphatic primary alcohols. We had successfully used microwave instrument for activating hydrolysis reaction of the wax ester in beeswax to policosanol in short time.**

Keywords—beeswax; hydrolysis; microwave; policosanol

I. INTRODUCTION

Policosanols (PC) are defined as a mixture of high molecular weight aliphatic primary alcohols with chain lengths ranging 20 to 36 carbon. The mainly of long chain alcohols are compost of docosanol, tetracosanol, hexacosanol, octacosanol and triacontanol [1]. Policosanol is one of component having the potential to be used in food application and pharmaceutical. Due to it had effect on intermittent claudication, reducing plated aggregation and LDL peroxidation and endothelial function and aiding in the prevention of cardiovascular disease [1-7]. The policosanol extracted from sugar cane and beeswax are major sources of raw materials in the products production [8].

Beeswax is a main source of policosanols. It is consist of mainly esters of fatty acids and various long-chain alcohols and several minor components such as triglyceride and free fatty acid. However, the content and compositions of policosanol in beeswax are various with origins, extraction and purification technique [8-11]. Irmak et al., 2006 [8] reported that the analyzation of beeswax from USA indicated aliphatic alcohol from C 20 to 30 and the main component of policosanols more than 40% is triacontanol (C30-OH) Whereas, Jimenez et al., 2004 [10] reported on the composition of beeswax from difference provinces of Spain which are compost of various of long chain aliphatic alcohol from 16 to 34 carbon. Miguel and Fernando, 2013 [11] shown beeswax from Portuguese in carbon 18 to 34 atoms.

The extraction of policosanol was divided into two main steps: Extraction and Purification. Policosanol present in beeswax can be extracted by hydrolysis or saponification reaction with NaOH or KOH base in various times and temperatures [8, 12-18]. Many researchers have investigate on the use of enzyme catalyst methanolysis in supercritical carbon dioxide [19], using High ultrasound (HIU) as catalyst [20]. For purification of policosanol have to use of organic solvents for washing [3, 8-9, 12-18, 21] and following by crystallization [22]. The most commonly technique for policosanol analyzation and characterization can be done by TLC, GC and HPLC or combining these technique [3, 15-16, 23-25].

The popular technique for hydrolysis the wax ester into long chain alcohols or policosanol and free fatty acids is refluxing technique. Moreover, all most of researchers have been using of various organic solvents such as n-hexane, toluene, benzene, ethyl acetate, ethanol, methanol and acetone in preparing the NaOH or KOH base for catalyst the reaction in long time of hydrolysis. Miguel, and Fernando [11] had been used KOH as catalyst in policosanol extraction at 100 ºC for 2 hours. Gamble and et.al, [22] report on beeswax extraction by hydrolysis with KOH in acetone at 50-60℃ for 3-7 hours following by cooling at 2 to -10 ℃ for 18 hours to purify the policosanol.

However, the time requirements is drawback for the policosanol extraction from beeswax by hydrolysis reaction. Thus, the main objective of this study is to development of policosanol extraction from beeswax in the short time-consuming by microwave radiation transmitting energy in activation the hydrolysis reaction. The efficiency of isolation and purification of aliphatic alcohols were done after beeswax hydrolysis following by the characterization of the alcohol by GC.

II. Materials and Method

A. Materials and reagents

Beeswax was obtained from bees farm in local area at Chiang Mai, Thailand. Chemical reagents in analytical grade were provided from LabScan (Bangkok, Thailand). Standard hydrocarbons and aliphatic long chain alcohols were supplied by Sigma Aldrich, Thailand. Commercial rice bran oil (as triglyceride) in food grade was purchased from local supermarket in Chiangmai, Thailand.

B. Moisture content, Melting point and composition of beeswax

The moisture content was determined by drying at 60°C until the weight constant.

The melting point was done by transfer the beeswax into the capillary tube and fixed with thermometer. The fixed capillary tube and thermometer was dropped into oil baths and heating the oil. The range of beeswax melting temperature was recorded in three times of repeating.

The compositions of beeswax were studied by using TLC technique under the mixture of hexane, ethyl acetate and acetic acid (90: 9: 1, v/v/v) as developing solvent and bands developed by iodine resublimed. The spot on TLC plate are compost of beeswax, rice bran oil (as triglyceride; TG), standard steric acid and standard steric acid methyl ester.

C. High purity beeswax preparation

Crude beeswax sample 20 g was dissolved in 140 ml of hexane and it was repeated 2 steps of refluxing. The first step, the temperature for refluxing beeswax was 65℃ for 30 minutes and following by washing with hot hexane in twice times. The second step, the beeswax was repeated by refluxing with isopropanol at 70℃ for 30 minutes. The get rid of impurities beeswax was checked by TLC plate and the bands developed as previous experiment.

D. Optimization of Potassium hydroxide concentration and organic solvent type for catalyst

The KOH solution were prepared in various concentration 0.5, 1.0 and 2.0 M in many type of solvents such as 80 % ethanol, toluene, hexane, chloroform and petroleum ether for catalyzing the reaction.

E. Policosanol extraction by microwave energy

The high purity beeswax was hydrolyzed with various ethanolic KOH concentration at 0.5,1.0 and 2.0 M which the reaction was activated by microwave as source of energy. The energy of microwave was varied at 100 watt for 5 minutes and 300 watt for 60, 90 and 120 seconds. The complete hydrolysis of beeswax was tasted by TLC in Chloroform: hexane: acetic acid, 70: 30: 1 (v/v/v) as developing solvents.

F. Policosanol purification

The complete hydrolyzed beeswax was purified by refluxing with 200 ml of the mixture of toluene, water and ethanol in ratio 70:20:1 (v/v/v). The refluxing was operated at 80-90 °C for 30 minutes following by decreasing temperature to 60-70 °C for 30 minutes. The toluene extracted layer was separated to purify by washing and overnight soaking with isooctane, water and ethanol (70: 20: 10 v/v/v). The mud cake layer was dried at 60 °C resulting in the purified policosanols.

G. Policosanol composition identification

The composition of policosanol from beeswax extraction was analyzed by capillary column of GC-FID and GC-MS. Policosanols samples were prepared by accurately weighting the proper amount the high purity policosanol after that it was dissolved with isooctane resulting in sample solutions.

GC-FID: GC (Agilent Technologies 7890A series) separations were performed using a fuse silica HP-5 capillary column (5% phenyl-95% diethylpolysiloxane, 30 mm 0.25 mm, 0.25 μm film thickness). The temperature was programed from 150 to 280 °C in 15 °C/min of rate and detector temperature was seted at 280 °C. The carried gas was nitrogen with a flow rate 1 ml/min and the detector temperature was 280 °C. The Pressures of hydrogen and air were 60 and 5 KPa, respectively. The injection volume was 1.3 μl and the split ratio 1:30

GC-MS: A Hewlett-Packard 6890 series gas chromatograph (GC) system was directly coupled to a Hewlett-Packard 5973 mass spectrometer. A fused silica capillary HP-5 from Hewlett-Packard used for the analysis. The GC oven temperature was kept at 50 °C for 1 minute and programed at 5 °C/min to 320 °C and maintained at this temperature for 15 minutes. The inlet temperature was 300°C. Initial flow rate of the carried gas (helium) was 1.0 ml/min. The MS-temperatures were as follow: ion source: 230°C, quadrupole: 150°C. The ionization energy was 70 eV and a mass range of 50-600 U (2 scans/s). The sample were injected with 1:10 split ratio. Data analysis was carried out by using HP Chemstation software. The peaks were also confirmed with NIST/EPA/NIH Mass spectra library.

III. Results and Discussion

A. Moisture content , melting point composition of beeswax and high purity beeswax preparation

Physical properties of crude beeswax which are moisture content and melting point. The results of moisture content shown in 0.4927±0.0857 % and the range of melting point had been 58-62℃ which is near to the data of Morgan et al, 2002 [26]. The beeswax material are compost of wax ester, triglyceride, free fatty acid and hydrocanbon (Figure 1.). which is closely to the report of Gemble and William, 2003 [22-24]. Thereby, the moisture content, free fatty acid and triglyceride were in case of rancidity. These impurity

substances had once effect on fairly broad range of the melting point apart from the mixture of various long chain primary aliphatic alcohols with various long chain fatty acids. Moreover, the removal of the impurities in beeswax such as triglycerides and free fatty acids is necessary process to prevent soap forming via hydrolysis with strong base (saponification).

The triglycerides and free fatty acids in beeswax were eliminate by refluxing with organic solvent. Figure 2. show the high purity of beeswax on TLC plate. We had been success in elimination of the triglyceride impurity thereby the remains of free fatty acid and hydrocarbons will be removed with long chain fatty acid by product in hydrolysis reaction. The high purity beeswax was kept for policosanol extraction.

Fig. 1. Thin layer chromatography of crude beeswax (1,Std. hydrocarbon; 2, rice bran oil (TG); 3, beeswax; 4, std. fatty acid methyl ester (FAME); 5, std. free fatty acid (FFA)).

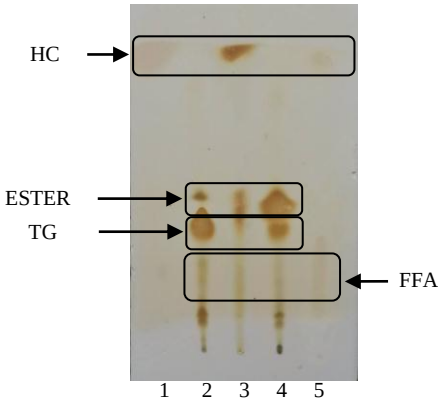
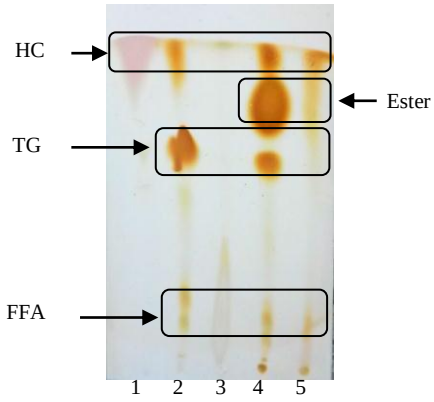


Fig. 2. Thin layer chromatography of high purity beeswax (1,Std. hydrocarbon; 2, rice bran oil (TG); 3, std. free fatty acid (FFA); 4, std. fatty acid methyl ester (FAME); 5, purified beeswax).



B. Optimization of Potassium hydroxide concentration and organic solvent type for catalyst

The concentration of KOH had effect to soluble efficiency in polar and non-polar organic solvents due to the simultaneous solution of the reaction is very importance. The data in table I shows that 80% ethanol is best indeed effective solvent for dissolving in all of the KOH concentrations studied. Moreover, the advantage of KOH solution in ethanol is to protection the possible emulsion formation of the reaction. The KOH solution in 80 % ethanol be prepared in 0.5, 1.0 and 2.0 M of concentration to use as catalyst for beeswax hydrolysis in policosanol extraction by microwave activation.

TABLE I. THE SOLIBILITY OF KOH IN VARIOUS CONCENTRATION AND ORGANIC SOLVENTS

KOH concentration (M)	Organic solvent/solibility				
	80% Ethanol	*Toluene*	*Hexane*	*Chloroform*	*Petroleum ether*
0.5.	++++	+	+++	+	++
1.0	++++	+	++	+	+
2.0	++++	+	+	+	+

The symbol (+) show the degree of KOH solubility.

C. Policosanol extraction by microwave energy

The TLC in Figure 3 had been present to hydrolysis of the high purity wax ester by microwave as source of energy at 100 watt for 5 minutes with 0.5, 1.0 and 2.0 M of KOH concentration. From the data, all of KOH concentrations show high efficiency in catalyzation the hydrolysis in only 5 minutes at 100 watt via microwave energy. The wax ester did not appear on the TLC plate the after hydrolysis of wax to long chain fatty acid and long chain aliphatic alcohol. However, the minimum base concentration (0.5 M KOH) display to completely catalyst the reaction by microwave activation.

Moreover, the use of microwave has proved to give faster by increasing electric power of microwave to 300 watt and decrease in periodicity of time to 60, 90 and 120 seconds. The results of high purity beeswax hydrolysis had shown in Figure 4 The wax ester were completely hydrolyzed in 60 seconds rapid time under 0.5 KOH concentration as catalyst.

Fig. 3. Thin layer chromatography of hydrolyzed beeswax (1,Std. fatty acid methyl ester (FAME); 2, Std. fatty alcohol; 3, std. free fatty acid (FFA); 4,5 and 6, hydrolyzed beeswax (100 watt, 5 mins) with 0.5, 1.0 and 2.0 M in 80%ETOH, respective.

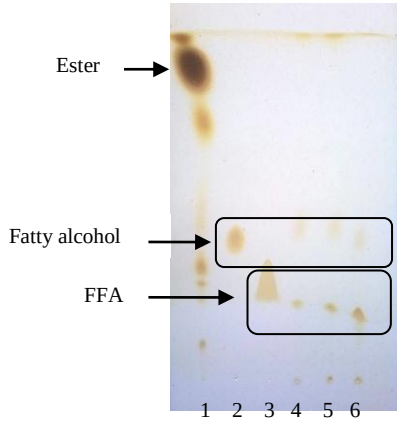
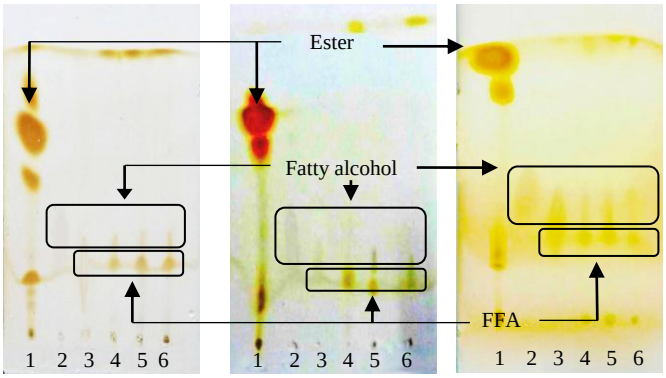


Fig. 4. Thin layer chromatography of high purity beeswax (1,Std. fatty acid methyl ester (FAME); 2, Std. fatty alcohol; 3, std. free fatty acid (FFA); 4,5 and 6, hydrolyzed beeswax (0.5 M in 80%ETOH) at 300 watt for 60, 90 and 120 sec.



D. Policosanol purification and composition identification

The aim of hydrolyzed wax ester purification is to elimination of long chain fatty acids. It was done by 2 step of temperature refluxing with the mixture of solvent. The high temperature in first step involves in non-policosanols elimination [21] and the high non-polar solvent in second step had affect to increasing of policosanols purity due to decreasing the free fatty acid solution in this phase. The policosanols purity was tested by TLC in Figure. 5 and the policosanols recovery was dried at 60 °C resulting in white – yellow powder using for GC analyzation. Furthermore, the quite good policosanol yield of the beeswax hydrolysis reaction activation by microwave shows 14.57%.

Fig. 5. Thin layer chromatography of high purity policosanol (1,Std. fatty alcohol.; 2, std. free fatty acid (FFA); 3,Std. fatty acid methyl ester (FAME),; 4, hydrolyzed beeswax; 5, toluene fraction;6, mud cake in isooctane fraction.

The purified policosanols was dissolved with isooctane for identification the composition of policosanol by GC-FID and GC-MS. The chromatogram of GC-FID analyzation shows seven peak of aliphatic long chain alcohol or policosanol components separated in present study, their identities are reported in Figure 6. Peaks assignment was performed via long chain alcohols standard thereby retention times (t_R) of the alcohols extracted were good agreement with the corresponding peaks in the standard alcohols. The long chain aliphatic alcohols extracted from beeswax were isolated in the following ratios: C18-OH, 0.12%; C20-OH, 0.42%; C22-OH, 1.34%; C24-OH, 21.94%; C26-OH, 17.24%; C28-OH, 20.01%; C34-OH, 38.95%. The data of retention time and percentage of long chain alcohols were summarized in table II.

GC-MS analysis of this mixture showed identifiable as five alcohols in Figure. 7. The policosanols compositions of the samples were identified by direct comparison of their chromatographic retention times and mass spectra with those of authentic compounds. The result had indicated that the data of GC-MS had correspond to the GC-FID data. However, the various policosanols composition depend on the origins of beeswax such as Asia, America, European or African zone [8-11].

TABLE II. RETENTION TIME AND PERCENTAGE OF LONG CHAIN ALCOHOLS COMPOSITION IN BEESWAX

Retention time (T_R)	Alcohol type	% Ratio
9.126	Octadecanol (C18-OH)	0.12
10.304	Eicosanol (C20-OH)	0.42
11.376	Docosanol (C22-OH)	1.34
12.471	Tetracosanol (C24-OH)	21.93
13.867	hexacosanol (C26-OH)	17.24
15.857	octacosanol (C28-OH)	20.01
18.913	Tetratriacontanol (C34-OH)	38.94
Total		100

Fig. 6. Chromatography of long chain primary aliphatic alcohol (A; Standard; B, extraction from beeswax).

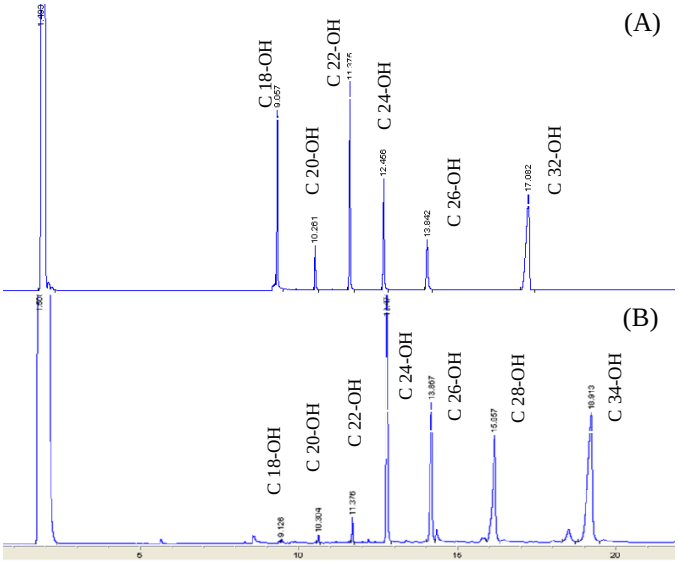
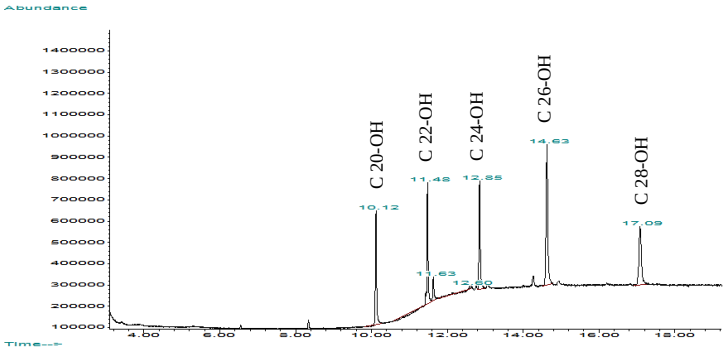


Fig. 7. Total ion chromatogram of fraction of aliphatics alcohol product from beeswax extracted.



IV. Conclusion

Beeswax were shown to be readily converted to their respective aliphatic long chain alcohols and long chain fatty acid by microwave energy in activation the hydrolysis reaction. The microwave radiation transmitting energy was found to be effective in the hydrolysis to high conversion yields of primary aliphatic long chain alcohols (policosanol) and following advantages over the currently refluxing method: speed, no special extraction instrumentation is needed.

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References

[1] S. Irmak, and N. T. Dunford, "Policosanol contents and compositions of wheat varieties," Journal of Agricultural and Food Chemistry, vol. 53, pp. 5583-5586, 2005.

[2] V. Fraga, R. Menendez, A.M. Amor, RM. Gonzalez, S. Jimenez, and R. Mas, "Effect of policosanol on in vitro and in vivo rat liver microsomal lipid peroxidation," Archives of Medical Research, , vol.28(3), pp. 355-360, 1997.

[3] .I. Gouni-Berthold, and H.K. Berthold, "Policosanol: clinical pharmaceuticology and therapeutic significance of a new lipid-lowering agent," American Heart Journal, vol. 143, pp. 356-365, 2002.

[4] S. Kato, K.I. Karino, S. Hasegawa, J. Nagasawa, A, Nagasaki, M. Eguchi, T. Ichinose, K. Tago, and H. Okumori, "Octacosanol affects lipid metabolism in rats fed on a high-fat diet," British Journal of Nutrition (London), vol. 73, pp. 433-441, 1995.

[5] S. Valdes, M.L. Arruzazabala, and E. Fernandez, "Effect of policosanol on platelet aggregation in healthy volunteers," International Journal of Clinical Pharmacology Research, vol. 16, pp. 67-72, 1996.

[6] F. Francini-Pesenti, F. Brocadello, D. Beltramolli, M. Nardi, L. Caregaro, "Sugar cane policosanol failed to lower plasma cholesterol in primitive, diet-resistant hypercholesterolaemia: a double blind, controlled study," American Journal of Health-System Pharmacy, vol. 60(11), pp. 1-5, 2003.

[7] S. Harrabi, S. Boukhchina., P. M. Mayer, H. Kallel, "Policosanol distribution and accumulation in developing corn kernels," Food Chemistry, vol. 115, pp. 918-923, 2009.

[8] S. Irmak, N. T. Dunford, and J. Milligan, "Policosanol contents of beeswax, sugar cane and wheat extracts," Food Chemistry, vol. 95 pp. 312-318, 2006

[9] B. Ilaria, and P.C. Maria, "Characterisation of beeswax in works of art by gas chromatography-mass spectrometry and pyrolysis-gas chromatography-mass spectrometry procedures" Journal of Chromatography A, vol. 1028, pp. 297-306, 2004.

[10] J.J. Jimenez, J.L. Bernal, S. Aumente, M.J.D. Nozal, M.T. Martin, and J.Jr. Bernal, "Quality assurance of commercial beeswax: I gas chromatography - electron impact Ionization mass spectrometry of hydrocarbons and monoester," Journal of Chromatography A, vol. 1024, pp. 147-154, 2004.

[11] M. Miguel, and M.N. Fernando, "Authentication of beeswax (Apis mellifera) by high-temperature gas Chromatography and chemometric analysis," Food Chemistry, vol. 136, pp. 961-968, 2013.

[12] N. T. Dunford, and E. Jeff, "Nutritional bioactive components of wheat straw as affected by genotype and environment," Bioresource Technology, vol. 101, pp. 422-425, 2010.

[13] O.C., Aicha, B.M, Mahamad, K, Belhassen, B. Sadok, P. Claude, and K. Habib,"Comparison of the concentration of long-chain alcohols (Policosanol) in three tunisian Peanut varieties (Arachis Hypogaea L.)", Journal of Agricultural and Food Chemistry, Vol 58, pp. 12143-12148, 2010.

[14] S. Irmak, R.S. Jonnala, and F. MacRitchie, "Effect of genetic variation on phenolic anid and policosanol contents of Pegaso wheat lines," Journal of Cereal Science, vol. 48, pp. 20-26, 2008.

[15] P. Adhikari, K.T. Hwang, J.N. Park, and C.K. Kom, "Policosanol content and composition in Perilla seeds," Journal of Agricultural and Food Chemistry, vol 54, pp. 5359-5362, 2006.

[16] K.T. Hwang, C.L. Weller, S.L. Cuppett, and M.A. Hanna, "Policosanol contents and composition of grain sorghum kernels and dried distillers Grains," Cereal Chemistry, vol. 81(3), pp. 345-349, 2004.

[17] N. Gladys, B. Paul, G.D. Micheline, P. Patrick, and H.L. Marie,"Composition of sugarcane waxes in rum factory wastes," Phytochemistry, vol. 61, pp. 721-726, 2002.

[18] M.F. Wang, H.L. Lian, L. Mao, J.P. Zhou, H.J. Gong, B.Y. Qian, Y. Fang, and J. Li, "Comparision of various extraction methods for policosanol from rice bran wax and eatablishment of chromatographic fingerprint of policosanol," Journal of Agricultural and Food Chemistry, vol 55, pp. 5552-2558, 2007.

[19] A.J. Michael, and J.E. Fred, "Isolation of long-chain aliphatic alcohols from beeswax using lipase-catalyzed methanolysis in supercritical carbon dioxide," Journal of Supercritical Fluid, vol. 37, pp. 173-177, 2006.

[20] G. Cravotto, A. Binello, G. Merizzi, and M. Avogadro,"Improving solvent-fee extraction of policosanol from rice bran by high density ultrasound treatment," European Journal of Lipid Science and Technology, vol. 106, pp. 147-151, 2004.

[21] N. T. Dunford, S. Irmak, and R. Jonnala, "Pressurised Solvent Extraction of policosanol from wheat straw, germ and bran," Food Chemistry, vol. 119, pp. 1246-1246, 2010.

[22] W.R. Gemble, Z. Liu, D.T. Bailey, P.P. Perez, D.P. Stull, S.L. Richheimer, R.L. Nochols, R, Lenobel, High molecular weight primary aliphatic alcohols obtained from natural products and Uses thereof, United States Patent, 2003.

[23] A.P. Tulloch, "Beeswax-composition and analysis," Bee World, vol. 61 (2), pp. 47-62, 1980.

[24] A.P. Tulloch, "Structure of the ester and their component hydroxyl acids and diols," Chemistry and Physics of Lipids, vol. 6 (3), pp. 235-265, 1971.

[25] Y. Lin, R. Mike, P.J.W. Reggy, A.T. Elke, M. Gerald, S. Aafje, and W.M. Gert, "Wheat germ policosanol failed to lower plasma cholesterol in subjects with normal to mildly elevated cholesterol concentrations", Metabolism, vol. 53 (10), pp. 1309-1314, 2004.

[26] J. Morgan, S. Townley, S. Kemble, and R. Smith, "Measurement of physical and mechanical properties of beeswax," Materials Science and technology, vol. 18, pp. 463-467, 2002.