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Extraction Purification and Composition Determination of Policosanols from Thai Beeswax

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ABSTRACT

Policosanol is a mixture of long chain aliphatic alcohol of 20-36 carbon atoms. It has been use in pharmaceutical composition and dietary supplements. Beeswax is a rich sources of Policosanols. In this research, we focus on extracted and purified policosanols from Thai beeswax. The compositions of beeswax were checked by TLC and it contains wax ester, free fatty acids and triglycerides. The impurity such as triglycerides and free fatty acid were removed via refluxing with hexane. The get rid of triglycerides beeswax was saponified with 2.0 molar NaOH in 80% ethanolic on 3 hours. The saponified beeswax was great purified by the mixture of toluene : water : ethanol (70 : 20 : 10 v/v) in first step and use of isooctane : water : ethanol (70 : 20 : 10 v/v) in the second step. The isooctane extracted mostly contains policosanols without free fatty acids. The policosanols compositions were identified by gas chromatography and the range of carbon chain in fatty alcohol were C_{18} to C_{30}. Thereby Tetracosanol (C_{24}) was predominant fatty alcohol in range 34.80 - 46.54 % proportion of total alcohols.

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INTRODUCTION

Policosanols (PC) are a mixture of long chain aliphatic primary alcohol (20-36 carbon) that have been reported to have low-density lipoprotein and increasing high-density lipoprotein (Castano *et al*., 2001, Mas *et al*., 1999, Menendez *et al*., 1999 and Nurhan *et al*., 2010). Policosanols has also beneficial effects on smooth muscle cell proliferation, reducing plated aggregation and LDL peroxidation (Fraga *et al*., 1997, Kato *et al*., 1995 Gouni-Berthold and Berthold, 2002, Irmak and Dunford, 2005, Valdes *et al*., 1996). Currently, a number of dietary supplements containing policosanols are commercially available in the world market. The majority of these products are prepared from sugar cane and beeswax extracts (Irmak *et al*., 2006).

Beeswax is a rich source of policosanols. It is a complex mixture of long-chain alkanes, alkenes, monoester, diesters, hydroxymonoester and fatty acid as well as several minor components. Much of ester in beeswax are contain palmitic, oleic and tetracosanoic acid ester of alcohols ranging in length from 12 to 36 carbons, Monoester of C_{24} to C_{34} alcohols are about 40% of the composition of beeswax (Jackson, and, 2006). The compositions and content of substance in beeswax are various with origins (European bees *Apos mellifera*, African bees *Apis mellifera adansonii* and Asiatic bees *Apis dorsata*, *Apis florea* e *Apis indica)* (Ilaria and Maria, 2004). Beeswax from difference provinces of Spain are compost of various of long chain aliphatic alcohol from 16 to 34 carbon (Jimenez *et al*., 2004) whereas, beeswax from Portuguese shown in carbon 18 to 34 atoms (Miguel and Fernando, 2013). Thereby, 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) (Irmak *et al*., 2006).

The aim of this work was to establish the policosanols composition of beeswax from northern area of Thailand due to it is a main location of bee farms in the Country. The isolation and purification of alcohols was done after bees wax saponified follow by the characterization of long chain alcohol by GC-MS and GC-FID**.**

Experimental:

Materials:

Beeswax was obtained from Supa bee Farm in local area at Chiang Mai, Thailand. All solvents were analytical grade (Lab scan, Thailand). Sodium hydroxide, potassium hydroxide steric acid and steryl alcohol (C18-OH) were supplied by Sigma Aldrich,

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Thailand. Rice bran oil, used as a triglyceride standard was food grade.

Moisture content and compositions of beeswax:

The moisture content of the beeswax was determined by drying at 105°C until the weight constant.

The compositions of beeswax were done by spotting of beeswax, rice bran oil (as triglyceride; TG), standard steric acid (as free fatty acid; FFA) and steric acid methyl ester (as fatty acid methyl ester; FAME) on TLC plate. The developing solvent was a mixture of hexane, ethyl acetate and acetic acid (90: 10: 2, v/v/v) and developed bands by iodine resublimed.

Samples preparation:

Beeswax were prepared dissolving in hexane (10 g wax/10 ml) and it was repeated 3 times refluxing in water bath for 30 minutes with continuous stirring and following by washing with hot hexane. Then, the mixture was dried at 55°C for 24 hours to eliminate hexane. The get rid of impurity in wax was spotted on TLC plated and developing as previous experimental method.

Extraction and purification of Plicosanols from beeswax:

Five grams of high purity wax ester was hydrolyzed by refluxing with 100 ml of 0.5, 1.0 and 2.0 M of NaOH or KOH in 80% ethanol for 3 hours. The sample mixtures of hydrolyzed wax were check by TLC every 1 hour. The developing solvent was a mixture of chloroform, hexane and acetic acid (70: 30: 0.1, v/v/v) and developed bands by iodine resublimed.

The saponified wax ester was purified by refluxing with 200 ml of toluene, water and ethanol (70: 20: 10 v/v/v). The two steps of refluxing condition were heat at 80-90 °C with continuous stirring for 30 minutes in the first step following by decreasing temperature to 60-70 °C for 30 minutes without stirring in the second step. The toluene extracted was collected for repeating purification by washing and overnight soaking with isooctane, water and ethanol (70: 20: 10 v/v/v). The mud cake was discard and the mixture of solvents were separated from the cake. After the mud cake was dried at 60 °C resulting in policosanols. The policosanols purity was checked by TLC.

Compositional analysis of Policosanols by GC-MS and GC-FID:

Sample solutions were prepared by accurately weighting the proper amount the policosanols samples obtained as described under extraction of policosanols from beeswax and it were mixed with isooctane as solvent.

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 (5% phenyl-95% diethylpolysiloxane, 30 mx 0.25 mm, 0.25 μm film thickness) 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. Initial flow rate of the carried gas (helium) was 1.0 ml/min. The inlet temperature was 300°C. 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 policosanols compositions of the samples were identified by direct comparison of their chromatographic retention times and mass spectra with those of authentic compounds. The peaks were also confirmed with NIST/EPA/NIH Mass spectra library.

GC-FID:

GC (Agilent Technologies 7890A series) separations were performed using a HP-5 capillary column. The oven temperature was programed as follow: the initial temperature was set at 150°C for 3 minutes, raise to 280 °C at 15°C/min and held at 280°C for 10 minutes. 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.

RESULTS AND DISCUSSION

Moisture content and compositions of beeswax:

The moisture content of beeswax show in range 0.5266±0.03%. The compositions of beeswax material are show in Fig. 1. It is compost of wax ester, free fatty acid and triglyceride which is similar to previous report (Gemble and William, 2003). The get rid of impurity in wax such as triglycerides and free fatty acids is necessary step to prevent soap forming via hydrolyzation with strong base (saponification).

The elimination of triglycerides and free fatty acids were done by dissloving beeswax in hexane and repeated 3 times refluxing for 30 minutes with continuous stirring following by washing with hot hexane. TLC plate of the elimination show in Fig. 2. Triglycerides can be removed in the third refluxing time and washing with hexane. However, the remains of free fatty acid will be eliminated together with long chain fatty acid which is by product from saponification reaction or the policosanols

purification step. The high purity of wax ester will be used as material to extract policosanols.

Extraction and purification of Policosanols from beeswax:

The TLC had been present to hydrolyzation of the high purity wax ester by saponification reaction with 0.5, 1.0 and 2.0 M of NaOH in Fig. 3 and Fig. 4 for KOH in 80% ethanol for 3 hours. From the data, both of the strong base show high efficiency in catalyzation the saponification in only 1 hour at the lowest concentration. The wax ester did not appear on the TLC plate due to hydrolyzation the wax to long chain fatty acids and long chain aliphatic alcohols. The base NaOH 0.5 M were chosen for catalysis in saponification due to the reason to protection the possible emulsion formation from KOH catalysis. Moreover, a shorter reaction times have benefited to keep away from the formation of a brown precipitate thus highlighting a partial sample degradation (Ilaria and Maria, 2004).

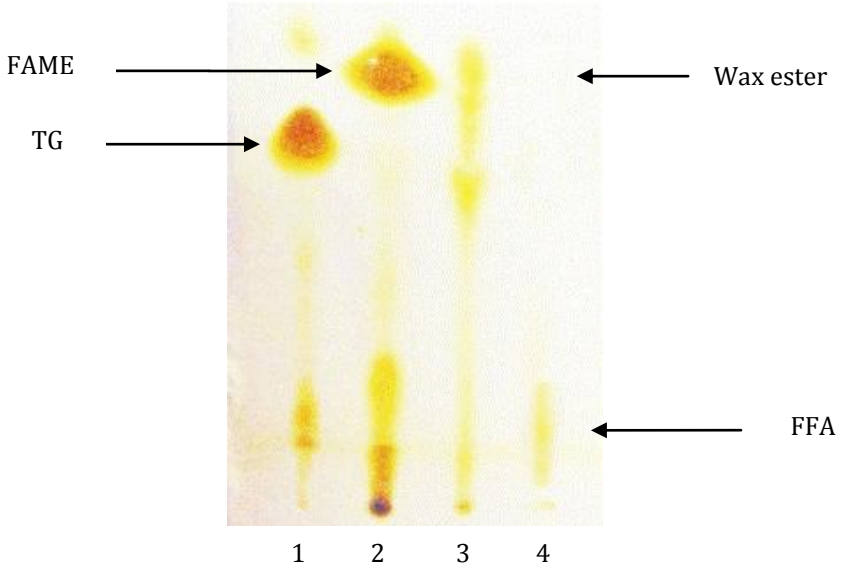


Fig. 1: Thin layer chromatography of beeswax sample materials. (1, rice bran oil (TG); 2, standard fatty acid methyl ester (FAME); 3, beeswax sample and 4, standard free fatty acid (FFA)).

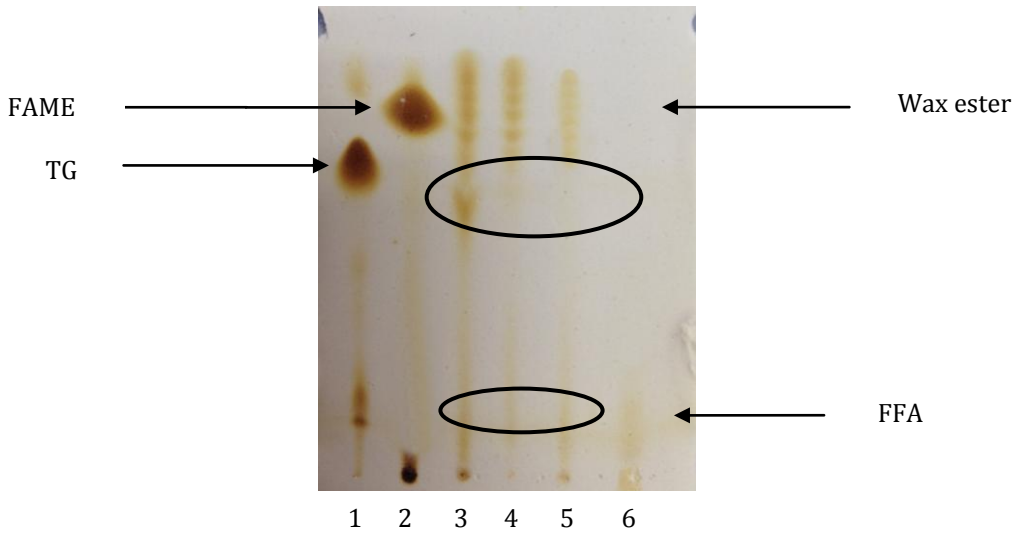
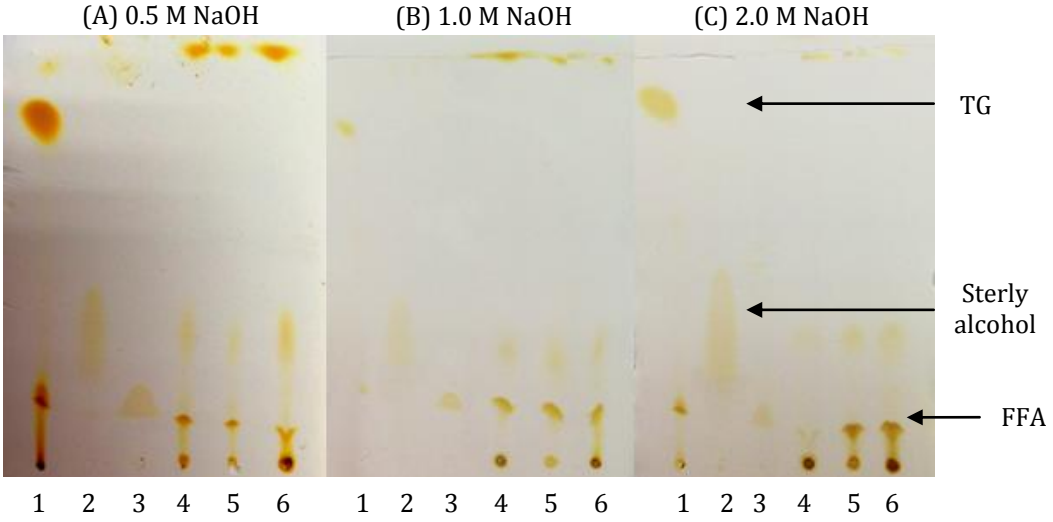


Fig. 2: Thin layer chromatography of the get rid of impurity from beeswax sample materials. (1, rice bran oil (TG); 2, standard fatty acid methyl ester (FAME); 3, first reflux beeswax; 4, second reflux beeswax; 5, third reflux beeswax and 6, standard free fatty acid (FFA))

The composition of long chain fatty acids and long chain aliphatic alcohols (Policosanols; PC) from saponified wax ester were purified by 2 step of temperature refluxing with the mixture of toluene, water and ethanol (70: 20: 10 v/v/v). The high temperature in first step at 80-90°C involves in non-policosanols elimination (Nurhan *et al.*, 2010). The policosanols dissolved in toluene whereas sodium

salt dissolved water in the fact that the emulsion forming of long chain hydrocarbon in fatty acid dissolving in non-polar toluene is a problem in purification. This problem can solved by ethanol addition into the solvent mixture, thereby clear of the layer separation. The policosanols extracted were repeated extraction via washing and soaking with isooctane water and ethanol (70: 20: 10 v/v/v). The non-polar isooctane which more than toluene had affect to increasing of policosanols purity due to decreasing the free fatty acid solution in this phase. The supernatant was collected to check by TLC which the indirectly way used to check policosanols purity (Fig. 5) and policosanols recovery got from this fraction by isooctane evaporation. The precipitate was dried at 60 °C resulting in white – yellow powder of policosanols for GC analyzation.



Figu. 3: Thin layer chromatography of saponified beeswax catalyzed by (A) 0.5, (B) 1.0 and (C) 2.0 M of NaOH in 80% ethanol. (1, rice bran oil; 2, steryl alcohol; 3, standard free fatty acid (FFA); 4, 1 hr. of hydrolyzation; 5, 2 hr. of hydrolyzation and 6, 3 hr. of hydrolyzation).

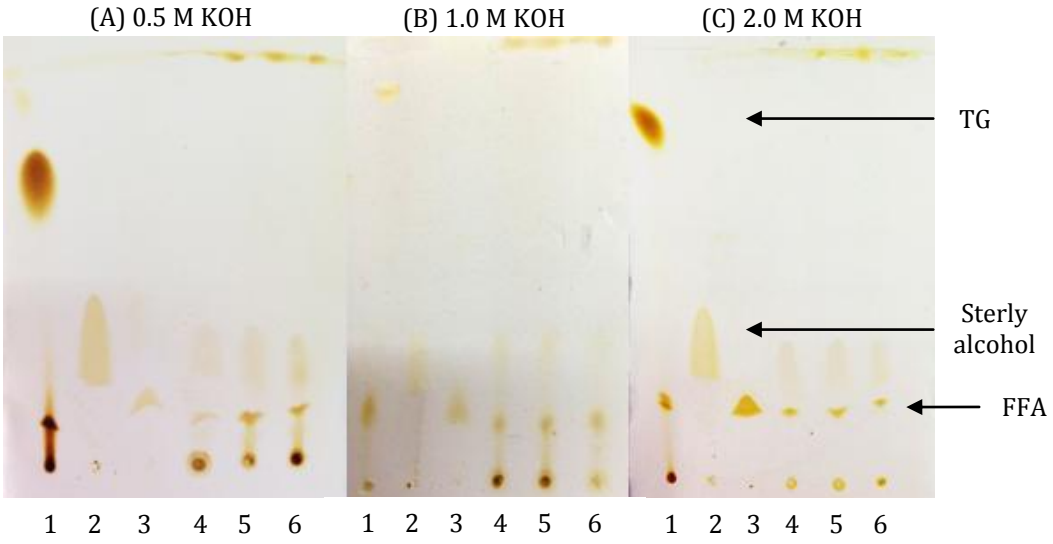


Fig. 4: Thin layer chromatography of saponified beeswax catalyzed by (A) 0.5, (B) 1.0 and (C) 2.0 M of KOH in 80% ethanol. (1, rice bran oil (TG); 2, steryl alcohol; 3, standard free fatty acid (FFA); 4, 1 hr. of hydrolyzation; 5, 2 hr. of hydrolyzation and 6, 3 hr. of hydrolyzation)

Compositional analysis of Policosanols by GC-MS and GC-FID:

Sample solutions were prepared by mixed the policosanols extracted with isooctane. GC-MS analysis of this mixture showed identifiable as five alcohols in Fig. 6 and the policosanols composition and content repeated analysis by GC-FID. A typical chromatogram of policosanols in beeswax sample is shown in Fig.7. Identification of the components and content are shown in table1. Beeswax from northern

area of Thailand is compost of various of long chain aliphatic alcohol from 18 to 30 carbon and the main component of policosanols more than 40% is Tetracosanol (C24-OH) (Irmak *et al*., 2006). The various policosanols composition depend on the origins of beeswax such as America, European or African Jimenez *et al*., 2004, Miguel and Fernando, 2013, Ilaria and Maria, 2004 and Irmak *et al*., 2006).

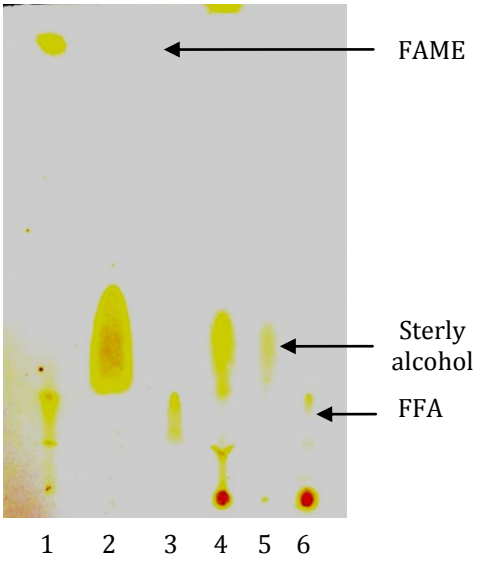


Fig. 5: Thin layer chromatography of the purify policosanols extracted (1, standard fatty acid methyl ester (FAME); 2, steryl alcohol; 3, standard free fatty acid (FFA); 4, saponified beeswax with 0.5 M NaOH 1 hr.; 5, isooctane fraction and 6, water and ethanol fraction)

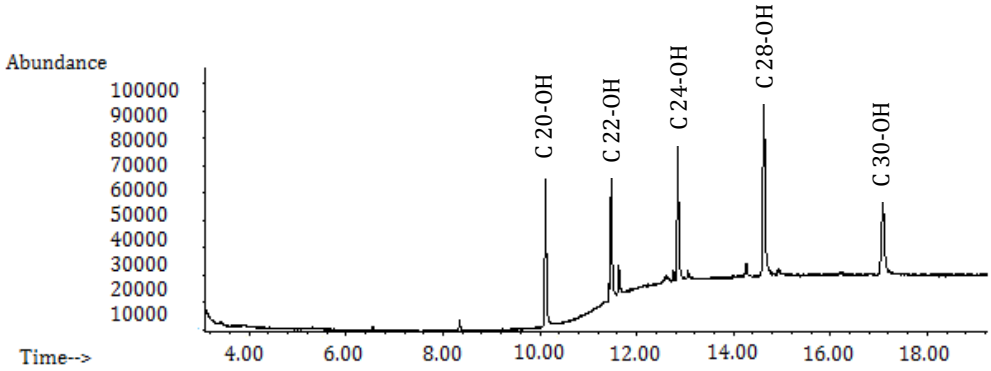


Fig. 6: Total ion chromatogram of fraction of policosanols product from beeswax.

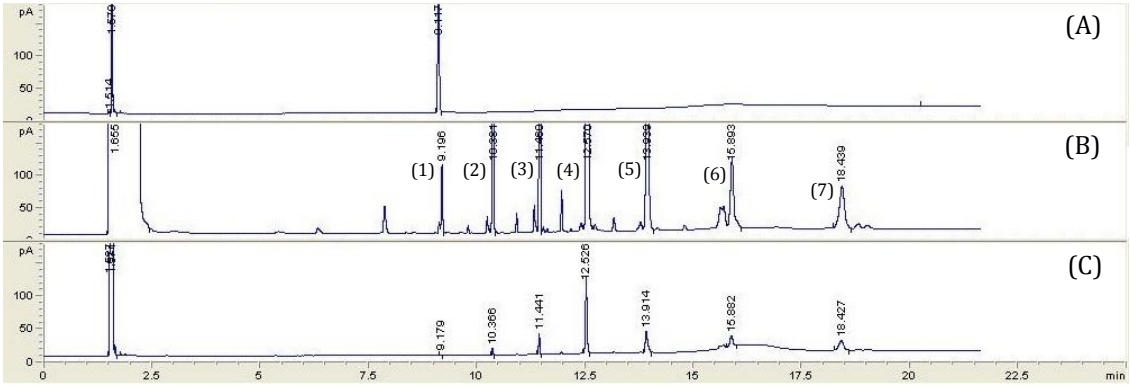


Fig. 7: Chromatograms of GC-FID of long chain aliphatic alcohol (A; steryl alcohol, B; supernatant fraction and C; precipitate fraction).

Table 1: Policosanols compositions of from beeswax.

Peak No.	Long chain alcohol	Long chain alcohol (% portion)
1	Octadecanol (C18-OH)	4.54
2	Eicosanol (C20-OH)	5.465
3	Docosanol (C22-OH)	10.54
4	Tetracosanol (C24-OH)	40.67
5	hexacosanol (C26-OH)	16.135
6	octacosanol (C28-OH)	9.515
7	Triacontanol (C30-OH)	13.135
Total		100

Conclusions:

In this work, a successful extraction, purification and determination of the chemical profile of policosanols from Thai beeswax was performed. Tetracosanol (C24-OH) have highest quantities of the long chain alcohols compared to the composition of beeswax. The different chemical compositions of policosanols in beeswax were relation to natural beeswaxes and source-dependent. This study exhibit a preliminary data of policosanols from beeswax in Asia for developing to pharmaceutical industries.

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