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Antihypertensive Activity and Phytochemicals Analysis of *Chassalia curviflora* Extracts

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Abstract

The objectives of this study are to determine the antihypertensive activities of leaves and flower of *Chassalia curviflora* and compare the potential between two different extraction methods which are hot water and methanol extract. The biuret protein assay was conducted to determine the protein protein concentration in samples. The phytochemical in leaves and flower extracts of *C. curviflora* were analyzed by using GC-MS. The result of protein concentration in *C. curviflora* flower was higher compared to leaves extract of 0.6648 mg/ml and 0.5431 mg/ml, respectively. The hot water extract of *C. curviflora* flower showed the highest antihypertensive activity with the percentage of ACE inhibitory activity of 95.50 ± 0.06% with IC_{50} value of 3.71 µg/ml. The 10 highest peak area (%) of phytochemical in all samples were: bis(2-ethylhexyl) ester (34.64 %), Cyclotrisiloxane, hexamethyl- (31.14 %), (Phenylthio)acetic acid, 1-adamantylmethyl ester (30.90 %), Hexanedioic acid, Cyclononasiloxane, octadecamethyl- (18.357 %), Oleic Acid (16.56 %), n-Hexadecanoic acid (15.23 % and 14.15 %), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (16.43 % and 12.98 %) and Trichloromethane (11.03 %). In conclusion, both of leaves and flower of *C. curviflora* have a potential as antihypertensive agent.

Key words: Leaves extract; Flower extract, ACE inhibitory, Gas Chromatography–Mass Spectrometry (GCMS).

Introduction

The treatment of hypertension mainly depends on synthetic medicines. There are several classes of drugs have been used to treat hypertension over the past forty years. The class of drugs includes beta blockers (β-blockers), diuretics and also angiotensin converting enzyme (ACE). The ACE inhibitors are correlated with a low rate of adverse side effects and it is the most preferred antihypertensive agent class for treating patients who suffered from hypertension. However, according to Atkinson and Robertson, (1979) the drugs have some negative effects[1].

Over thousands of years, natural products have played the important role in prevention of disease and health care. Phillipson (2001), stated that the ancient civilization from Indians, Chinese and also North Africans use natural sources for curing many types of diseases[2]. Recently, there are much herbal medicines which use traditionally for treating hypertension. According to Fernão C. et. al. (2007), a number of compounds from plants has been analyzed to have potential *in vitro* ACE inhibitory activity[3]. The compounds are; phenylpropanes, flavonoids, xanthones, fatty acid, terpenoids, alkaloids oligosaccharides and peptide amino acids. *C* is one of the species which

have potential for ACE inhibitory activity. Nowadays, there are many types of plants which have potential to produce antihypertensive agents. As the antihypertensive agent is used for the treatments of the hypertension disease, *C. curviflora* which may have potential of antihypertensive action was being investigated.

Materials and Methods

Collection of Plant Material

Leaves and flower of *C. curviflora* were collected from Northern Malaysia, specifically in Kedah, Malaysia.

Preparation of plant extract

Methanol extract. The fresh leaves and flowers of *C. curviflora* were dried for two weeks. The samples were homogenized by using the grinder machine until the fine powders were formed. A methanol extraction, the method was adopted from Dupont et al., (2006) with some modification[4]. A total of 50 g of each samples were separately weighed and immersed into 1000 ml of methanol with regular shaking at ambient temperature for 72 hours. The extracts were filtered by using Whatman filter paper No. 1 and the filtrates were evaporated by using rotary evaporator until it was dried.

Hot water extract. An extraction method was adopted from Mhd Omar et al., (2011) with slightly modification[5]. A total of 50 g of each samples were immersed in 500 ml distilled water with the ratio of 1:10 and boiled for 30 minutes. The filtrates were filtered by using Whatman No.1 filter paper and freeze dried for 3 days to remove the water.

The samples were labeled as LHWE; Leaves hot water extract, LME; Leaves methanol extract, FHWE; Flower hot water extract and FME; Flower methanol extract.

Biuret Protein Assay

The biuret reagent was conducted by mixing 0.6 g of Cupric Sulphate ($CuSO_4.5H_2O$) with 1.8 g sodium potassium tartrate ($NaKC_4H_4O_6$) and dissolved in 120 ml 0.2 N of NaOH solution followed by addition of 1.0 g Potassium Iodide (KI). The resulting solution was then transferred into a 250 ml beaker and diluted to the total volume of 200 ml with distilled water. The reagent must be discarded if precipitation formed. The samples were weighed out 0.35g and added with 10 ml of 0.5% deoxycholate (SDS). The mixtures were vortexed for 1 minute and centrifuged at 2500 rpm for 30 minutes. Two ml of biuret reagent was added into each tube and all of the tubes were covered with parafilm and slowly vortex to mix well. All of the tubes were incubated at room temperature for 15 minutes and the absorbance was read at 550 nm.

Antihypertensive Assay

Antihypertensive activities of *C. curviflora*. leaves and flower extracts were determined using a kit (ACE kit- WST from Maryland, USA). Enzyme working solution was prepared by adding 1.5 ml of Enzyme B solution to Enzyme A. The Enzyme B solution is the mixing of Enzyme B with 2 ml of deionized water. Indicator enzyme solution was prepared. Both Enzyme C and Coenzyme were dissolved with 3 ml deionized water respectively. A volume of 2.8 ml of each Enzyme C solution and Coenzyme were added into Indicator solution. Sample solution was prepared by dilute a stock solution (0.01 µg/ml) with deionized water for the concentration of 1/5, $1/5^2$, $1/5^3$, $1/5^4$, $1/5^5$ and $1/5^6$. The dilution sample of 10 µl was added with 10 µl Substrate buffer and 10 µl of enzyme working solution. The mixture was incubated for one hour at 37°C. Then, 200 µl of the indicator working solution was added and incubate again for 10 minutes at room temperature. The absorbance was read at 450 nm by using spectrophotometer. ACE inhibitory activity of each sample was recorded and analyzed. IC_{50} (50% inhibitory concentration) was determined using the inhibition curve from the ACE inhibitory activity. The ACE inhibitory activity was calculated using the following equation:

ACE inhibitory activity (inhibition rate %) = [(Ablank 1 – Asample) / (Ablank 1 – Ablank 2)] x 100

Gas Chromatography Mass Spectrometry (GCMS) Analysis

The samples were analyzed according to J. Jamilah et al., (2012) with some modification as follow: DB-1MS fused-silica column (30m X 250µm X 0.25µm) was used[6]. The oven temperature was initially set at 60°C and held for 1 min and then programmed with the rate of 10°C/min and hold at 310°C for 5 min resulting in the complete elution of all peaks analyzed. The Helium was used as a carrier gas at constant flow 1 ml/min with the split ratio of 1:50 with the injection temperature 250°C. The transfer line temperature was 310°C while the detector temperature 230°C. Total GC running time was 32 min. The extracted sample was diluted with the ratio 1:10 and filtered using the syringe filter so that the sample was diluted enough to be injected into the inlet of GC-MS.

Statistical Analysis

The results were expressed as the mean ± standard error of the mean (S.E.M), analyzed by SPSS 20.0. Differences between groups were tested by one-way analysis of variance (ANOVA). The P values less than 0.05 were considered statistically significant. All *in vitro* assays were performed in triplicates.

Results and Discussion

Antihypertensive is closely related to peptides which are major component of protein. The presence of protein compound was firstly detected. The concentration of protein in leaves and flower of *C. curviflora* were depicted in Table 1. The known quantities of the analyte were responding when an analytical method is approach. Both leaves and flower extracts of *C. curviflora* showed high protein concentration of 0.64 mg/ml and 0.68 mg/ml, respectively. According to Boyer (2000), compounds

consist of two or more peptide bond react with the biuret reagent will form a purple coloured complex[7]. Switzer & Garrity (1999), also reported that the colour product was the result of the coordination complex of copper atom and two nitrogen atoms from each peptide chain[8]. The maximum intensity for the colour reached was after 15 minutes incubation and the complex will stable for several hours.

The percentages of ACE inhibitory activities against concentrations (µg/ml) were depicted in Figure 1. The percentages of ACE inhibitory activities of all extracts were proportionally elevated as the concentration increased. At concentration of $1/5^6$ µg/ml, the FHWE showed the highest percentage of inhibition while LHWE gave the lowest percentage of 9.22 ± 0.75% and 1.75 ± 0.15%, respectively. The percentage of ACE inhibitory activities of FME was 6.14 ± 0.43% while for the LME was 2.27 ± 0.46%. The FHWE was significantly higher compared to LME, LHWE and FME at concentration from $1/5^6$ µg/ml to $1/5^3$ µg/ml.

The inhibition activities continuously increased at the concentration of $1/5^2$ µg/ml and 1/5µg/ml. The LME showed the highest percentage of ACE inhibitory activity at $1/5^2$ µg/ml of 80.94 ± 0.02 % but not significantly different compared to FHWE of 79.59 ± 0.25%. The LHWE showed the lowest percentage of inhibition of 39.84 ± 1.12 %. Nugroho et al. (2013), has studied about *Anacardium occidentale* L. (*Anacardiacees*), one type of cashew species said that extract of cashew leaves exhibited antihypertensive effect, which was certainly correlated to active chemical compounds contained in the extract[9]. Extract of cashew leaves mainly contains phenolic and flavanoid compounds. Some epidemiology studies showed inverse correlation between flavanoid consumption and risk of cardiovascular disease[10]. It also suggested that antihypertensive effect of extract of cashew leaves might be related to the present of the compounds

Overall, all the samples showed dose dependent manner activities. The more ACE can be reduced, the higher the activity of inhibition in *C. curviflora*. According to Meunieret et al. (1987) and Jonadet et al. (1990), aqueous extraction method extracted the anthocyanins, one of the major group of compounds present in this plant which could be bioactive compounds producing different antihypertensive action mechanisms such as the inhibition of angiotensin I and angiotensin II converting enzyme[11,12]. This may be the reason hot water extraction more higher compared to methanol extraction

The IC_{50} values (µg/ml) of the extracts were depicted in Table 2. According to Aprilita Rinayanti et, al. (2013), the IC_{50} was defined as the concentration of extract required to reduce 50% of ACE activity, which was determined by analysis of ACE inhibitory (%) against extract concentration[13]. From Table 2, the IC_{50} was inversely proportional to the percentage of ACE inhibitory activities (%). FHWE showed the lowest IC_{50} value (3.39 µg/ml) while LHWE had highest IC_{50} value (4.96 µg/ml). Messerli (2002), who studied about antihypertensive of *P. yezoensis* stated that the lower IC_{50} value indicates the strongest ability as antihypertensive and the activity as it would be expected not to have the side effects associated with synthetically drugs production which used to control hypertension[14].

Thus, both extracts possessed ACE inhibitor properties. However, the flower of *C. curviflora* has a higher value of ACE inhibition activities compared to leaves of *C. curviflora*. This also associated with protein concentration in the flower compared to the protein concentration in the leaves of *C. curviflora*. Samova et. al. (2003), suggested that ethanolic extracts of plant named *O. africana* may exert the effect of antihypertensive through the modulation of the sympathetic nervous system or by using the ACE inhibitor[15]. In addition, chemical analysis from Osim et. al. (1999), showed the aqueous extract of roots and stems of *Olea* possessed ACE inhibitor properties[16]. Traditionally, *Chassalia* sp. was used by folks to treat the other diseases besides treating the antihypertensive, but less scientific study carried out to prove

the activity. According to Gustaffson et. al. (1994 and 2000), *Chassalia* sp. has recommended for wound dressing, liver disease and also imflammation[17,18]. In addition, *Chassalia* sp. was used as an insect repellent while for the HIV inhibitory, the macrocylic peptides have been isolate from the tropical tree *Chassalia parvifolia*.

The phytochemicals of flower and leaves of *C. curviflora* were analysed by using GCMS to investigate the compounds which may relate to the antihypertensive activity. The analyses of six samples of *C. curviflora* were carried out and 20 major compounds with higher peak area were recorded. The comparison of peak area (%) between the 6 samples which were; raw flowers (RF), flowers hot water extract (FHWE), flowers methanol extract (FME), raw leaves (RL), leaves hot water extract (LHWE), and leaves methanol extract (LME) of *C. curviflora* were depicted in Table 3.

The major compounds in all the samples identified were n-Hexadecanoic acid, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydoxy-6-methyl-, Hexanedioic acid, bis(2-ethylhexyl) ester, Hydrazine, 1,1-dimethyl-, Oleic acid, Trichloromethane, .gamma.-Sitosterol2-Furancarboxaldehyde, 5-methyl-, 2-Furanmethanol, 2-Methoxy-4-vinylphenol, 9,12-Octadecadienoic acid (Z,Z)-, Acetophenone, Campesterol, Cyclotrisiloxane, hexamethyl-, Eicosane, Octadecanoic acid, and Stigmasterol. However, the compound with highest peak area was Hexanedioic acid, bis(2-ethylhexyl) ester (34.64%) in FME with retention time at 21.53 and the compound with lowest peak area was Stigmast-4-en-3-one (1.22%) is common compound for steroid.

The n-Hexadecanoic acid is the highest compound found in RF (15.23%), FHWE (3.64%), RL (14.15%), LHWE (3.88%) and also LME (5.4%) of *C. curviflora.* According to Akhilesh & Anusha (2013), n-Hexadecanoic acid was found to act as antioxidant, hypocholesterolemic, nematicide, hemolytic and also 5-alpha reductase inhibitor. Antioxidant may relate to the antihypertensive[19]. This was proved by research of Anthoney et.al. (2013), stated that good correlation has been found between antihypertensive and antioxidant activity by DPPH assay which the antioxidant activity can contribute to prevent the increase of blood pressure[20]. Thus, this compound might play important roles during the ACE inhibition activities.

The phytol compound of 3.05% (data was not shown) which available in RL function for antidiabetic, anti-angeogenic, antimicrobial, anticancer, anti-inflammatory and also antidiarrhoeal. Besides, Vitamin E was also can be found in RL (2.22%) at retention time 26.78. Even though vitamin E only small amount in the sample, it gave lots of activities. Akhilesh and Anusha (2013), reported that vitamin E which from compound methylated phenols can contribute to antioxidant activity, antiatherogenic, antithrombotic, anticoagulant, neuroprotective, and antiviral. Besides, vitamin also play important role in immune-modulatory, cell membrane-stabilizing and antiproliferative actions. As antioxidant has strong correlation to antihypertensive, this compound may also involved during the ACE inhibitory activities.

Conclusion

In sum, leaves and flower of *C. curviflora* possessed antihypertensive properties. Flower extract of *C. curviflora* showed the significant level of percentage of ACE inhibitory activities (%) compared to leaves extract. Thus, the IC_{50} of hot water extract of *C. curviflora* flower showed the lowest value and inversely proportional to the ACE inhibitor activity. Phytochemicals analysis of samples gave the 10 highest compounds were; bis(2-ethylhexyl) ester (34.64 %), Cyclotrisiloxane, hexamethyl- (31.14 %), (Phenylthio)acetic acid, 1-adamantylmethyl ester (30.93 %), Hexanedioic acid, Cyclononasiloxane, octadecamethyl- (18.357 %), Oleic Acid (16.56 %), n-Hexadecanoic acid (15.23 % and 14.15 %), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (16.43 % and 12.98 %) and Trichloromethane (11.03 %). The

potential of *C. curviflora* deserves further assessment as a therapeutic agent for the treatment of hypertension in humans.

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TABLES AND FIGURES

Table 1: The protein concentration of leaves and flower of *C. curviflora.*

Sample	Concentration of protein (mg/ml)
Leaves	**0.64**
Flower	**0.66**

Table 2: The IC_{50} (µg/ml) of extracts of ACE inhibitory activities

Sample	IC_{50} value (µg/ml)
LHWE	4.96
LME	4.06
FHWE	3.39
FME	3.71

* LHWE; leaves hot water extract, LME; leaves methanol extract, FHWE; flower hot water extract, FME; flower methanol extract.

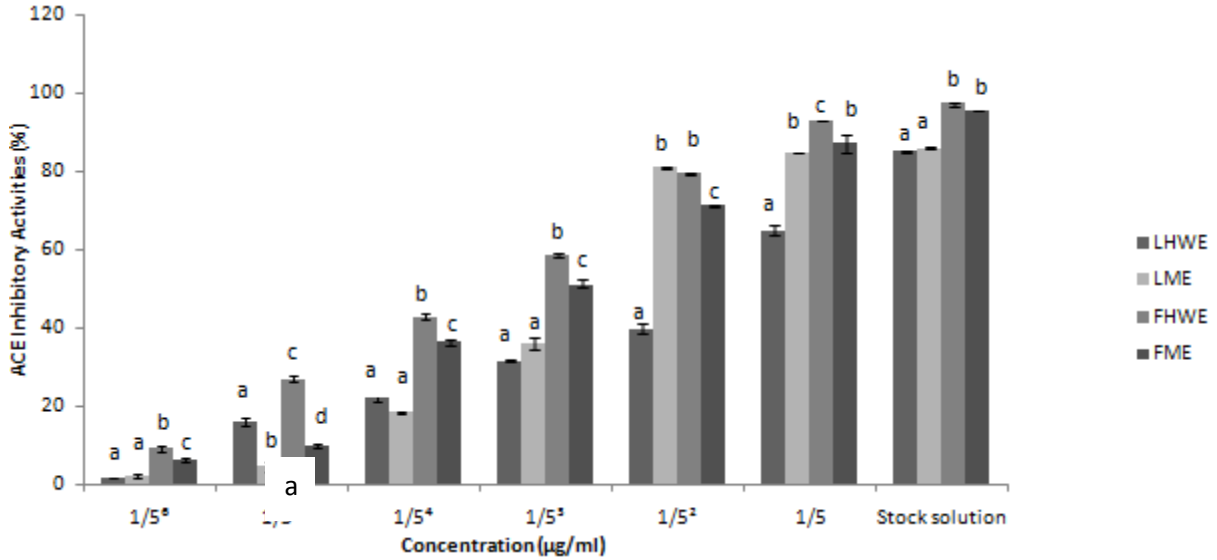

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Figure 1: The graph of ACE inhibitory activities (%) between leaves and flower of *C. curviflora* extracts by concentration.

Mean with different superscript were significantly different ($P < 0.05$) leaves and flower between hot water and methanol extracts. Values expressed were mean ± S.E.M of triplicates. The concentration of stock solution used was 0.01 µg/ml

* LHWE; leaves hot water extract, LME; leaves methanol extract, FHWE; flower hot water extract, FME; flower methanol extract.

Table 3 : Phytochemicals analysis of *C. curviflora* leaves and flower in percentages of peak area (%)

Name of compounds	Molecular formula	Peak Area (%) of the samples					
		RF	FHWE	FME	RL	LHWE	LME
(Phenylthio)acetic acid, 1-adamantylmethyl ester	$C_{19}H_{24}O_2S$			30.93			
gamma.-Sitosterol	$C_{29}H_{50}O$	8.11			6.21		
1,1,1,3,5,5,5-Heptamethyltrisiloxane	$C_7H_{22}O_2Si_3$						5.71
1,13-Tetradecadiene	$C_{14}H_{26}$				1.26		
1,19-Eicosadiene	$C_{20}H_{38}$				5.73		
1,3,5,7-Tetraethyl-1-ethylbutoxysiloxycyclotetrasiloxane	$C_{14}H_{38}O_6Si_5$		1.58				
1,5,9-Decatriene, 2,3,5,8-tetramethyl-	$C_{14}H_{24}$					2.52	
1H-Imidazole, 1,5-dimethyl-	$C_5H_8N_2$					3.18	
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	$C_6H_8O_3$		1.98				
2-Furancarboxaldehyde, 5-methyl-	$C_6H_6O_2$		2.19			1.51	
2-Furanmethanol	$C_5H_6O_2$		2.19			3.52	
2-Hexadecene, 3,7,11,15-tetramethy	$C_{20}H_{40}$				1.67		
2-Methoxy-4-vinylphenol	$C_6H_8O_3$		1.42			2.02	
2-Propenamide	C_3H_5NO		1.69				
3,4-Difluoroanisole	$F_2C_6H_3OCH_3$					2.29	
3-Acetonylcyclopentanone	$C_8H_{12}O_2$	1.75					

3-Pyridinecarboxylic acid, 5-ethenyl-, methyl ester	$C_{10}H_{12}ClNO_2$	1.49					
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$		16.43		1.99	12.98	3.07
5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	$C_{12}H_{20}O$					1.43	
5-Hydroxymethylfurfural	$C_6H_6O_3$					8.92	
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	5.04	1.27				
9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	$C_{30}H_{50}O$				2.72		
9,19-Cyclolanost-24-en-3-ol, acetate, (3.beta.)-	$C_{32}H_{52}O_2$	2.2					
9-Octadecyne	$C_{18}H_{34}$				3.32		
Acetophenone	$C_6H_5C(O)CH_3$		4.59			1.48	
Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$						8.35
Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$						2.81
Benzenamine, 4-(2-phenylethenyl)-N-(3,5-dimethyl-1-pyrazolylmethyl)-	$C_{20}H_{21}N_3$			3.3			
Benzeneacetaldehyde	C_8H_8O		2.12				
Benzofuran, 2,3-dihydro-	C_8H_66O		3.35				
Benzoic acid	$C_7H_6O_2$					2.04	
Bicyclo[11.3.0]hexadecane-2,14-dione	$C_{16}H_{26}O_2$				2.29		
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	$C_{10}H_{18}$						1.35

Campesterol	$C_{28}H_{48}O$	4.86			3.73		
Cyclohexanol, 3,5-dimethyl-	$C_8H_{16}O$						2.13
Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$					18.357	
Cyclopentanone, 2-cyclopentylidene	$C_{10}H_{14}O$					3.69	
Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$			31.14			11
Docosane	$C_{22}H_{46}$	1.95					
Eicosane	$C_{20}H_{42}$				2.7	1.86	
Heptacosane	$C_{27}H_{56}$	1.9					
Hexacosane	$C_{26}H_{54}$	2.28					
Hexacosane, 9-octyl-	$C_{34}H_{70}$	1.69					
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	1.44					
Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$		5.4	34.64		1.55	
Hexasiloxane, tetradecamethyl-	$C_{22}H_{42}O_4$		1.3				
Hydrazine, 1,1-dimethyl-	$C_2H_8N_2$		8.61		1.35	3.97	
Lanosterol	$C_{30}H_{50}O$				1.35		
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.23	3.64		14.15	3.88	5.4
Nonadecyl trifluoroacetate	$C_{21}H_{39}F_3O_2$	3.08					
Nonane, 4-methyl-5-propyl-	$C_{13}H_{28}$		1.25				
Octadecanoic acid	$C_{18}H_{36}O_2$	2.61			3.11		
Oleic Acid	$C_{18}H_{34}O_2$	8.7			16.56	1.91	

o-Toluic acid, 2-tetrahydrofurylmethyl ester	$C_{13}H_{16}O_3$				1.96	
Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-henei cosapentaenyl)-, (all-E)-Squalene	$C_{30}H_{50}$		4.91			
Pentacosane	$C_{25}H_{52}$	3.41				
Phytol	$C_{20}H_{40}O$			3.05		
Pyrimidine, 2,4-difluoro-6-dimethylamino	$C_4H_4N_2$		4.2			
Salicylic acid	$C_7H_6O_3$				1.34	
Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$					5.07
Silver butanoate	$C_4H_7AgO_2$		2.89			
Stigmast-4-en-3-one	$C_{29}H_{48}O$			1.22		
Stigmasterol	$C_{29}H_{48}O$	3.31		4.06		
Tetracosane	$C_{24}H_{50}$	3.33				
Triacontyl acetate	$C_{33}H_{66}O_2$	3.81				
Trichloromethane	$CHCl_3$		15.73	2.19	11.03	
Trifluoroacetoxy hexadecane	$C_{18}H_{33}F_3O_2$	3.3				
Tris(tert-butyldimethylsilyloxy)arsine	$C_8H_2O_2Si$					5.37
Vitamin E	$C_{29}H_{50}O_2$			2.22		

*RF; raw flower, FHWE; flower hot water extract, FME; flower methanol extract, RL; raw leaves, LHWE; leaves hot water extract, LME; leaves methanol extract.