Genetic verification and chemical contents identification of *Allamanda* species (Apocynaceae)

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Abstract: *Allamanda* species (Apocynaceae) are popular ornamentals. Additionally, *A. cathartica* possesses medicinal properties whereas all other species have not been reported. This research aims to analyze genetics and chemical contents of *Allamanda* species existing in Thailand. The explored species are *A. blanchetii*, *A. cathartica*, *A. neriifolia*, *A. schottii*, and *A. violacea*. The dendrogram constructed from 16 inter-simple sequence repeat markers clearly distinguished species with genetic similarity values of 0.92-0.93 for species level and 0.50-0.76 for genus level. Diverse chemicals content in hexane extracts from *A. blanchetii*, *A. neriifolia*, *A. schottii*, and *A. violacea* were detected by gas chromatography-mass spectrometry. A high amount of squalene was found in *A. blanchetii* (55.81%) and *A. violacea* (51.09%). This content may function as a chemo preventative substance to protect people from cancer. α-Tocopherol, a form of vitamin E, was one of the predominant components found in *A. violacea* (26.325%), *A. schottii* (15.41%), and *A. neriifolia* (9.16%). One more substance, 9,12,15-octadecatrien-1-ol, was found to be relatively high in *A. schottii* (17.31%) and *A. neriifolia* (15.51%). Other minor and unknown compounds were also detected. The discovery of these chemicals provides an alternative and supplement for improving human well-being and pharmaceutical industries with natural resources, especially in light of the population increase.

Keywords: *Allamanda* (Apocynaceae); gas chromatography-mass spectrometry; genetic similarity; inter-simple sequence repeat

INTRODUCTION

Allamanda, a genus belongs to the family Apocynaceae, possesses 14 species native to tropical America (Middleton, 1999). The species have been popular ornamentals, because of their year-round production of large, bright yellow and purple flowers. *Allamanda* species have become naturalized throughout tropical climates, including in Thailand. Thailand is reported to have two native species, *A. cathartica* and *A. schottii* (Middleton, 1999) but various introduced species with diverse flower colors have long been popularly cultivated for ornamentation on roadsides and fences. Due to their fast growth in open, sunny areas with adequate rainfall, many species are cultivated as groundcover and for hedges and screens.

Allamanda cathartica is a wild spread and well-known species, used as both ornamental and medicinal plants. The plant contains allamandin which is a toxic iridoid lactone in all parts. Plumericin, isoplumericin, plumieride, and long chain esters are contained in the stem, root-bark, leaves, and roots. In addition, its leaves and stem yield lactones, ursolic acid, β-amyrin, β-sitosterol, and triterpenes. The root contains the antileukaemic iridoid lactone and three other iridoids: allamandin, allamandicin, and allamdin. The petals give off flavonoids, namely kaempferol and quercetin. The milky sap possess antibacterial and anticancer properties. Moreover, there are claims that distilled extract of the plant curing malignant and fungal and bacterial infectious diseases. Medicinally, its bark, leaves, and flowers relieve vitiated pitta, inflammation, constipation, ascites, and headache by external application. *In vitro* studies have supported its usefulness against human carcinoma cells of nasopharynx. *In vivo*, ethanol root extract has shown activity against P-388 leukemia in mice (http://archive.is/JlwpS, http://ayurvedicmedicinalplants.com/plants/1276.html, http://www.asianplant.net/*Apocynaceae*/Allamanda_cathartica.htm).

There are reports showing that the active chemicals in *A. cathartica* are allamandin, which is a toxic iridoid lactone, and cathartic, which is a defecated substance (Nayak *et al*., 2006; Islam *et al*., 2010; http://www.absoluteastronomy.com/topics/Allamanda). *Daphnis nerii* larvae died when fed by its leaves, perhaps due to variations in foliar nutritional and allelochemical factors (Hwang and Feng, 2001). Thai medicinal recipes have included small amounts of its leaves, bark, and milky sap as traditional medicines for laxative and inducing vomiting. When used in excess, however, it becomes a strong laxative and causes over-vomiting and sometimes death (Pupattanapong, 1979).

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Curiously, other species in the genus besides *A. cathartica* have never been reported for their chemicals or medicinal properties. Therefore, they should be characterized starting from the genetic relationships in the genus and preliminary chemical identification. DNA fingerprints based on inter-simple sequence repeat (ISSR) analyses are generally used to effectively indicate genetic relationships, useful for identification, conservation, sustainable uses, and plant breeding (Emel, 2010; Arslan, 2011; Gradzielewska, 2011). The banding patterns reveal genetic variation/relationship by cladogram construction. Homology, a concept critical to cladistics, can be defined as a similarity/distance resulting from common ancestry (Simpson, 2006). These genetic variation markers are commonly independent of environmental factors and are more abundant than phenotypic characters. Therefore, they provide a clearer description of the inherent variations in the studied genome.

For chemical identification of the plant extracts gas chromatography-mass spectrometry (GC-MS) has been successfully used in several plant group. For examples, Taweechaisupapong *et al*. (2010) identified constituents in *Boesenbergia pandurata* and found geraniol and camphor as the major compounds. Nadir *et al*. (2013) found 78 unreported constituents from *Salvia santolinifolia* by GC-MS analysis and other methods and identified that the species belongs to α-pinene chemotype.

This research aims to revise existing species diversity, verify genetics of the species group, and identify chemical contents.

MATERIALS AND METHODS

In 2010 the species of *Allamanda* were explored throughout Thailand and collected. The plants were identified by the authors followed Middleton (1999) and other literatures. Genetics and chemical contents were analyzed in the observed species. *Plumeria alba* was included as an outgroup for phylogenetic analysis.

Genetic analysis

All collected samples were subjected to DNA extraction and DNA fingerprinting by ISSR method. Total genomic DNA was extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience) then checked by using 0.8% agarose gel electrophoresis with tris-acetate (TAE) buffer and ethidium bromide staining. DNA samples were diluted to a final concentration of 20 ng/μL for use as DNA templates in the polymerase chain reactions (PCRs).

Amplifications were carried out on each *Allamanda* species, in 25 μL reaction mixtures consisting of GoTaq Green Master Mix (Promega), 0.5 μM primer, and 20 ng DNA template. Thirty two ISSR primers were screened, the 16 primers that successfully amplified clear bands were as follows (5′ to 3′): $(ACTG)_4$, $(AG)_7AAG$, $(AG)_8C$, $(AG)_8G$, $(AG)_8T$, $(CA)_6AG$, $(CA)_6GT$, $(CA)_8CC$, $(CA)_9A$, $(CA)_9G$, $(CA)_9T$, $(CT)_8TG$, $(GA)_6GG$, $(GT)_6CC$, $(GT)_6GG$, and $CCCC(GT)_6$. The reaction mixtures were incubated at 94°C for 3 min then amplified with the following 35 thermal cycles: denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, extension at 72°C for 2 min, and followed by a final extension at 72°C for 7 min on a Swift Maxi Thermal Cycler (Esco Micro Pte. Ltd.). Amplification products were detected by using 1.2% agarose gel electrophoresis and ethidium bromide staining then photographed. The amplified ISSR bands were scored as absent (0) and present (1) bands and these 0-1 data were used for dendrogram constructions using NTSYSpc 2.10p software (Rohlf, 1998).

Chemical constituent analysis

The extracts were prepared, and the chemical contents were analyzed by GC-MS. A 25 g sample of fresh leaves was ground, mixed with 120 mL hexane solvent (analytical grade), and filtered at room temperature. The filtrate was stored at -20°C until being analyzed by the GC-MS.

The GC-MS analysis of the crude extracts was performed using an Agilent Technologies GC 6890 N/5973 inert MS fused with a capillary column (30.0 m x 250 μm x 0.25 μm). Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injection and mass-transferred line temperature were set at 280°C. The oven temperature was programmed for 70°C to 120°C at 3°C/min, then held isothermally for 2 min, and finally raised to 270°C at 5°C/min. A 1 μL aliquot of the crude extract was injected in the split mode. The relative percentage of the crude constituents was expressed as the percentage using peak area normalization. Identification of the components of the crude was assigned by a comparison of the mass spectra obtained with those of the reference compounds stored in the Wiley 7N.1 library.

RESULTS

Species investigation and identification

The investigation of *Allamanda* in all regions of Thailand recognized five species (fig. 1) namely *A. blanchetii*, *A. cathartica*, *A. neriifolia*, *A. schottii*, and *A. violacea* (vouchers A. Chaveerach 756-760, kept at Department of Biology, Faculty of Science, Khon Kaen University, Thailand). They are all widely distributed throughout 76 provinces of Thailand as commercial, cultivated, and ornamental plants.

ISSR fingerprint analysis

The 16 different polymorphism primers successfully produced 1443 bands ranging in size from 300-3000 bp (fig. 2). ISSR banding patterns were used for dendrogram constructions. The dendrogram (fig. 3) shows the high power efficiency of the ISSR data used, which clearly distinguishes the outgroup *P. alba* from the ingroup. Also,

it separates each species on different branches. The identical species show genetic similarity levels of 0.92 (*A. violacea*) to 0.93 (*A. cathartica*), while the different species show levels of 0.50 (*A. schottii* and *A. cathartica*) to 0.76 (*A. blanchetii* and *A. violacea*).

These values are meaningful according to the phylogenetic tree, which separates each species into different monophyletic groups. In addition, these values are in accordance with the principle provided by Weier *et al*. (1982). that operational taxonomic units (OUT) show 0.85-1.00 similarity level should be recognized as the same species and 0.65 similarity level should be the same genus. However, ultimate interpretation of the dendrogram is dependent upon the taxonomist's knowledge of the OTU. ISSR banding data using similarity indexes supports five different species.

Chemicals constituent in the four studied species, identified by GC-MS

The preliminary phytochemicals screening on the hexane crude extract of the four *Allamanda* species shows the presence of different chemical compounds. The plants, their chemical compounds, retention time, and relative content are displayed in table 1. The total ion chromatographs (TIC) showing the peak identities of the compounds in the four individual species are given in fig. 4.

DISCUSSION

This exploration of *Allamanda* species diversity in Thailand found five species, two of which are native to Thailand (*A. cathartica* and *A. schottii*), and three of which are introduced species, popularly cultivated for year-round flower production (*A. blanchetii*, *A. neriifolia*, and *A. violacea*).

The molecular study, a subset of genomic study, used 1-2 individual species. These studies usually use much smaller sample size than in morphological studies, often as small as a single individual (Hillis, 1987). Analyses of large sample sizes are often limited by the availability of specimens, the expense of the analysis, and its time-consuming nature. ISSR showed the most powerful data, suitable for *Allamanda* species analysis. The dendrogram clearly distinguishes between identical species with 0.92-0.93 genetic similarity values and different species with 0.50-0.76 genetic similarity values.

As there are many publications revolving chemicals in *A. cathartica*, this research focused on chemical identification in the four other *Allamanda* species. A high amount of squalene was found in *A. blanchetii* and *A. violacea*, at 55.81% and 51.09%, respectively, and a minor amount was found in *A. neriifolia*, at 6.08%. All plants, animals, and humans produce squalene which is a triterpene. In the human body, it is a natural and essential component for the syntheses of cholesterol, steroid hormones, and vitamin D. It may be also an anticancer substance as it possesses a chemopreventative activity (Smith, 2000; Owen *et al*., 2004).

α-Tocopherol, a form of vitamin E that can be absorbed and accumulated in humans (Rigotti, 2007), is one of the predominant components of three out of four species, including *A. violacea*, *A. schottii*, and *A. neriifolia* at 26.33%, 15.41%, and 9.16%, respectively.

A final substance, 9,12,15-octadecatrien-1-ol, was found at relatively high levels in *A. neriifolia* (15.51%) and *A. schottii* (17.31%), however, its activity has never been reported.

In conclusion, the hexane crude extracts revealed some beneficial chemicals in the four *Allamanda* species. The discovery of such chemicals in these and other plant species can provide an alternative and supplement for improving human well-being and pharmaceutical industries with natural resources, especially in light of the population increase.

Table 1: Genetic similarities of *Allamanda* species, analyzed using DNA fingerprint data from 16 ISSR primers

	A. cathartica 1	*A. cathartica* 2	*A. violacea* 1	*A. violacea* 2	*A. blanchetii*	*A. neriifolia*	*A. schottii*	*P. alba*
Allamanda cathartica 1	1.00							
A. cathartica 2	0.93	1.00						
A. violacea 1	0.69	0.72	1.00					
A. violacea 2	0.66	0.69	0.92	1.00				
A. blanchetii	0.67	0.65	0.74	0.76	1.00			
A. neriifolia	0.65	0.63	0.59	0.62	0.67	1.00		
A. schottii	0.52	0.50	0.53	0.56	0.60	0.59	1.00	
Plumeria alba	0.36	0.32	0.32	0.37	0.46	0.44	0.55	1.00

Table 2: Chemical constituents of studied *Allamanda* species

Plant	RT (min)	Compound	Formula	MW	Relative content (%)
Allamanda blanchetii	55.094	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene	$C_{30}H_{50}$	410	55.810
	18.466	1,3-Ditert-butylbenzene	$C_{14}H_{22}$	190	6.012
		Unknown			38.179
A. violacea	55.100	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene	$C_{30}H_{50}$	410	51.087
	62.139	(2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol	$C_{29}H_{50}O_2$	430	26.325
	44.207	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	6.997
	44.671	Octadecanoic acid	$C_{18}H_{36}O_2$	284	4.730
	40.852	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	4.089
	29.659	Phenol,2,4-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	2.217
		Phenol,bis (1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	206	
		Unknown			4.555
A. neriifolia	44.213	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	15.509
		9,12,15-Octadecatrienoic acid	$C_{19}H_{32}O_2$	292	
		Ethyl (9Z,12Z)-octadeca-9,12-dienoate	$C_{20}H_{36}O_2$	308	
		Cis-11,14,17-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	320	
	62.139	(2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol	$C_{29}H_{50}O_2$	430	9.156
	61.699	Icosane	$C_{20}H_{42}$	282	8.653
		Hentriacontane	$C_{31}H_{64}$	437	
	56.740	Eicosane	$C_{20}H_{24}$	282	8.300
		Nonacosane	$C_{29}H_{60}$	408	
		2-Methylnonadecane	$C_{20}H_{42}$	282	
	44.665	Octadecanoic acid	$C_{18}H_{36}O_2$	284	7.100
	68.291	24(z)-Methyl-25-homocholesterol	$C_{29}H_{50}O$	414	6.709
		(3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5S)-5-Ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
	55.088	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene	$C_{30}H_{50}$	410	6.083
	40.852	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.138
	29.500	2,6-Bis(1,1-dimethylethyl)-4-methylphenol	$C_{15}H_{24}O$	220	3.836
	51.910	Hexacosane	$C_{26}H_{54}$	366	2.321
	48.772	Tetracosane	$C_{24}H_{50}$	338	2.061
		Hentriacontane	$C_{31}H_{64}$	437	
		Tetratriacontane	$C_{34}H_{70}$	479	
	40.699	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	1.986
	18.472	1,3-Ditert-butylbenzene	$C_{14}H_{22}$	190	1.835
	29.653	Phenol,2,4-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	1.364
		Phenol, bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	
	47.115	Tricosane	$C_{23}H_{48}$	324	1.348
		Hentriacontane	$C_{31}H_{64}$	437	
		Tetratetracontane	$C_{44}H_{90}$	619	
	47.386	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	290	0.761
		Unknown			17.841

continued…

Table 2: Continue

Plant	RT (min)	Compound	Formula	MW	Relative content (%)
A. schottii	44.225	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	17.311
		9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292	
	68.309	(3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5S)-5-Ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{29}H_{50}O$	414	15.406
		24(z)-Methyl-25-homocholesterol	$C_{29}H_{50}O$	414	
		(22R,24S)-22,24-Dimethylcholesterol	$C_{29}H_{50}O$	414	
		17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{29}H_{50}O$	414	
		1,5-Dimethyl-6(1,5-dimethylhexyl)-15,16	$C_{28}H_{46}O_2$	414	
	62.139	(2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol	$C_{29}H_{50}O_2$	430	9.131
	50.376	Pentacosane	$C_{25}H_{52}$	352	6.205
		Triacontane	$C_{30}H_{62}$	422	
		Docosane	$C_{22}H_{46}$	310	
		Octacosane	$C_{28}H_{58}$	394	
		Nonacosane	$C_{29}H_{60}$	408	
		Hentriacontane	$C_{31}H_{64}$	437	
		Hexacosane	$C_{26}H_{54}$	366	
		Tetracosane	$C_{24}H_{50}$	338	
		Hexatriacontane	$C_{36}H_{74}$	507	
		Icosane	$C_{20}H_{42}$	282	
		Carbonmonoxide; N, 1-diphenyl-1-pyridin-2-ylmethanimine; iron	$C_{21}H_{14}FeN_2O_3$	398	
		Tricosane	$C_{23}H_{48}$	324	
	40.846	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.762
	48.778	Tetracosane	$C_{24}H_{50}$	338	4.518
		Docosane	$C_{22}H_{46}$	310	
		Tetratriacontane	$C_{34}H_{70}$	479	
		Octacosane	$C_{28}H_{58}$	394	
		Henicosane	$C_{21}H_{44}$	296	
		Tricosane	$C_{23}H_{48}$	324	
		Carbonmonoxide; N, 1-diphenyl-1-pyridin-2-ylmethanimine; iron	$C_{21}H_{14}FeN_2O_3$	398	
		Pentacosane	$C_{25}H_{52}$	352	
		Icosane	$C_{20}H_{42}$	282	
	35.059	Henicosane	$C_{21}H_{44}$	296	4.341
		Heptacosane	$C_{27}H_{56}$	380	
	51.910	Hexacosane	$C_{26}H_{54}$	366	3.939
		Icosane	$C_{20}H_{42}$	282	
	39.777	Tetratriacontane	$C_{34}H_{70}$	479	3.753
		Heptacosane	$C_{27}H_{56}$	380	
	29.030	Docosane	$C_{22}H_{46}$	310	3.663
	47.110	Tricosane	$C_{23}H_{48}$	324	3.026
		Octacosane	$C_{28}H_{58}$	394	
		Icosane	$C_{20}H_{42}$	282	
		Docosane	$C_{22}H_{46}$	310	

continued…

Table 2: Continue

Plant	RT (min)	Compound	Formula	MW	Relative content (%)
		Henicosane	$C_{21}H_{44}$	296	
		Pentacosane	$C_{25}H_{52}$	352	
	53.396	Heptacosane	$C_{27}H_{56}$	380	2.911
		Icosane	$C_{20}H_{42}$	282	
		Unknown			20.035

Fig. 1: Observed *Allamanda* species throughout Thailand. A) *A. cathartica*; B) *A. violacea*; C) *A. blanchetii*; D) *A. neriifolia*; E) *A. schottii*

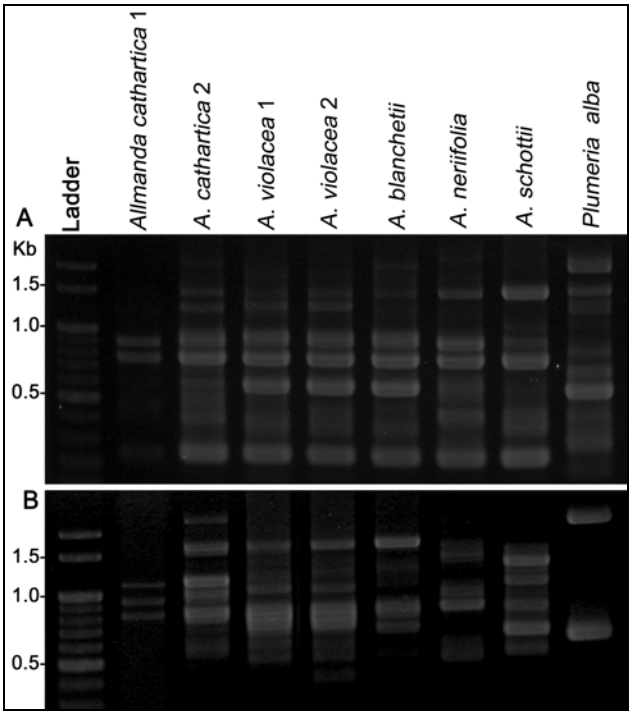

Fig. 2: Examples of ISSR banding patterns. a from primer $(GT)_6CC$; b from primer $(ACTG)_4$

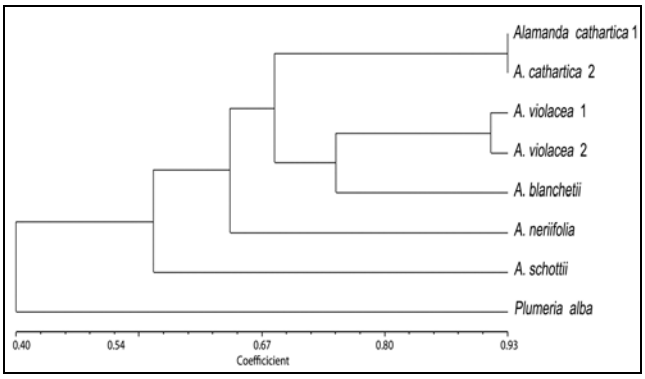

Fig. 3: The dendrogram constructed from ISSR bands from 16 primers of five *Allamanda* species by NTSYSpc 2.1p software

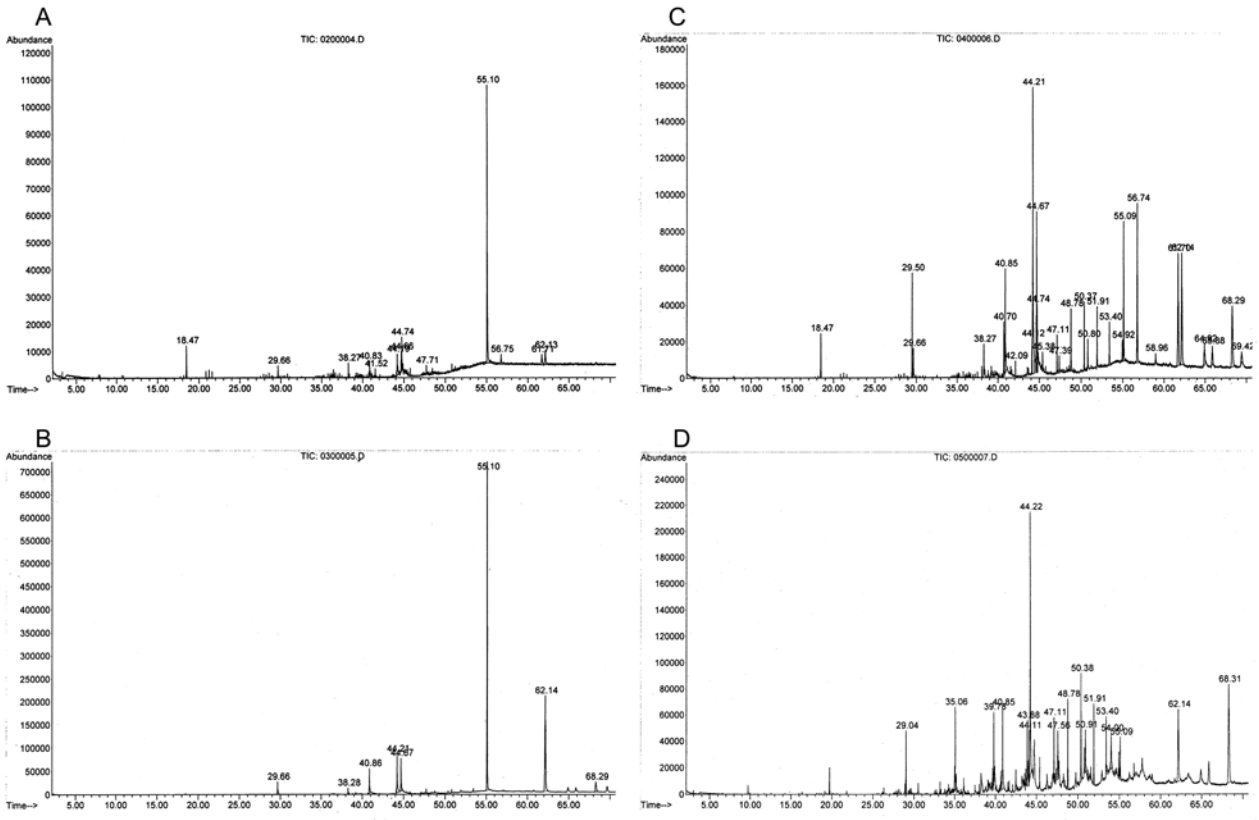

Fig. 4: GC-MS chromatogram of hexane crude extracts from leaves of the four *Allamanda* species. A) *A. blanchetii*; B) *A. violacea*; C) *A. neriifolia*; D) *A. schottii*

ACKNOWLEDGEMENT

This work is partially supported by research fund from Faculty of Science, Khon Kaen University.

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