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GC-MS Analysis of *Strobilanthes crispus* Plants and Callus

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Abstract

Strobilanthes crispus or locally known as "bayam karang", "pecah kaca", "jin batu" and "pecah beling" in Malaysia, has been traditionally used to increase immune system, treating kidney stones, treatment of diabetes mellitus, treatment of high blood pressure and treatment of wound. Studies examining the phytochemical constituents reported that the leaves of this plant contain ester glycosidic compound of caffeic acid, ρ-voumaric acid, , vanilic acid, ferulic acid, syringic acids, sitosterol, campesterol, hexadecanoic acid, methylester, lupeol, phytol, stigmasterol, flavonoid compounds such as (+)-catechin, (-)-epicatechin, rutin, and etc. While most of the literatures focused on the chemical compounds present in the leaves of *S. crispus*, none have been reported for the phytochemical constituents of the whole *S. crispus* plant including the leaf, stem, root or flower part. Besides, there is also lacking report on the tissue culture generated from this plant too. Thus, this study was carried out to profile the leaves, stems and roots and callus cultures of *S. crispus* using gas chromatography mass spectrometry (GC-MS) approach. Results revealed that this plant is rich with squalene, phytosterols such as stigmasterols and derivatives, sito-sterol, campesterols, as well as triterpenoids such as lupeol, amyrin and betulin.

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Introduction

Strobilanthes crispus or locally known as "bayam karang", "pecah kaca" , "jin batu" and "peach beling" in Malaysia and daun "picah belling" in Jakarta or "enyoh kelo", "kecibeling" or "ngokilo" in Java, is native to countries from Madagascar to Indonesia. This plant has traditionally been used by indigenous people in Perak, Malaysia to increase immune system. In order to do so, fresh leaves of *Strobilanthes crispus* are masticated and swallowed. Apart from that, leaves of *S. crispus* has also been used to treat kidney stones. This practice is carried out by heating the leaves of this plant and placing them on the hips (Ong & Norazlina, 1999). In addition, many others traditional usages of this plant have been reported such as for the treatment of diabetes mellitus, treatment of high blood pressure and treatment of wound (Yaacob *et al.,* 2010; Backer & Bakhuizen, 1963). This indicates that this plant contains compounds with pharmacological activities which could be used in drug development.

Studies examining the phytochemical constituent of *S. crispus* found that this plant contains polyphenols, catechins, alkaloids, caffeins, tannins, vitamins (C, B1, and B2) and high mineral content such as potassium (51%), calcium (24%), sodium (13%), iron (1%) and phosphorus (1%)

(Maznah *et al.*, 2000). It is also reported that leaves of this plant contain ester glycosidic compound of caffeic acid (a verbascoside), ρ-voumaric acid, caffeic acid, vanilic acid, ferulic acid, syryngic acids (Soediro et al., 1983). Besides, eight flavonoid compounds have also been identified by using HPLC from the leaves of *S. crispus*, which are (+)-catechin, (-)-epicatechin, rutin, myricetin, luteolin, apigenin, naringenin and kaempferol (Liza *et al.*, 2010).

Apart from that, Muslim *et al.* (2010) have also examined the phytochemical constituents of the methanolic and aqueous extracts of *S. crispus* dried leaves using GC-TOF mass spectroscopy approach and managed to identify 32 compounds such as 3-octadedecyne, α-sitosterol, campesterol, hexadecanoic acid, methylester, lupeol, phytol and stigmasterol in the methanolic extract while in aqueous extract, 3,5-dithiahexanol, 5,5-dioxide, cyclobutanol, hydrazine carboxamide, monoethanolamine, n-prophyl acetate and undecane and etc have been identified.

While most of the literatures focused on the chemical compounds present in the leaves of *S. crispus*, none have been reported for the phytochemical constituents of the whole *S. crispus* plant (leaf, stem, root, flower). Besides, there is also lacking reports on the tissue culture generated from this plant. Milne (1993) has stated that knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents, but also because such information may be of great value in disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and for discovering the actual significance of folkloric remedies. Hence, a thorough validation of the herbal drugs has emerged as a new branch of science emphasizing and prioritizing the standardization of the natural drugs and products because several of the phytochemicals have complementary and overlapping mechanism of action.

Therefore, this study was carried out to profile the whole *S. cripsus* plant from leaves, stems and roots and also the callus cultures induced from *S. crispus*'s leaves using gas chromatography mass spectrometry (GC-MS). Mass spectrometry, coupled with chromatographic separations such as Gas chromatography (GC/MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a powerful method for the analysis of non polar compounds and volatile essential oil, fatty acids, lipids. It was believed that the application of GC-MS in this study could yield good detection and identification of phytocompounds from *S. crispus* plant and its callus cultures.

Methodology

Preparation of Plant Extracts

Fresh plants of *S. crispus* plants were purchased from herbal supplier in Kota Kinabalu, Sabah. The plant was verified by a botanist from the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. A voucher specimen (ACSC 001/2013) was deposited in the herbarium of the same institute. The plants were thoroughly cleaned and the stems were cut into 15 cm long and re-cultivate

in soil enriched with humus for one year. Then, five one-year old plants were harvested, washed thoroughly and freeze-dried. The dried leaves, stems and roots were then grounded into powder form using a heavy duty blender. Plant powder was dissolved in absolute methanol at the ratio of 1:10. The mixtures were placed in a rotary shaker for 4 days at temperature 25°C and 180 rpm. The mixture was then filtered and concentrated under reduced pressure in a rotational evaporator. Then, the extracts were freeze-dried and stored at -80°C until further analysis.

Preparation of callus extract

Fresh leaves of purchased *S. cripus* plants were used as explants to induce the callus. The leaves were cut into 1 cm X 1 cm size and placed onto Murashige Skoog (MS) agar media that containing 1mg/L of NAA (1-Naphthaleneacetic acid) and 1 mg/L of BAP (6-Benzylaminopurine). The explants were left to grow in dark condition at 25°C for six months. Each media plate that contained six induced callus will be considered as one biological replicate. In this study, three biological replicates were used. The six callus in each plate were harvested together by washing away the attached agar, dried by using paper towel, ground into fine form using mortar and pestle. Then, the callus were weighed and dissolved in absolute methanol at the ratio of 1:10. The mixtures were placed in a rotary shaker for 4 days at temperature 25°C and 180 rpm. The mixture was then filtered and concentrated under reduced pressure in a rotational evaporator. Then, the extracts were directly subjected to GC-MS analysis.

GC-MS Analysis

The dried extracts were dissolved in HPLC grade methanol to appropriate concentrations for GC-MS analysis. A 1 µl of extract was injected into a GC-MS (GC model 7890, MS model 5975C, Agilent Technologies, Santa Clara, CA) after filtered with 0.22 µm pore size syringe filter. GC separation was performed on a HP-5MS capillary column (Agilent Technologies, Santa Clara, CA) operating at electron impact mode at 70 eV. Pure helium gas with built-in purifier was used at a constant flow rate of 1 ml/min employed in a splitless mode with injector temperature 250°C and ion source 280°C. The stepped temperature program was as follows: initial temperature oven was started at 220°C and hold for 5 minutes and followed by a ramp to 300°C at 5°C/min hold for another 15 minutes. A post-run of 5 minutes at 300°C was sufficient for the next sample injection. Mass analyzer was used in full scan mode scanning from m/z 40-550 and mass spectra were taken at 70 eV. For the compound identification, manual spectral matching was ascertained by using the mass spectral library of National Institute Standard and Technology (NIST) version 2.0 and with the aid of Automated Mass Spectral Deconvolution and Identification (AMDIS) software version 2.70 by deconvoluting the chromatography peak at the corresponding retention time.

Results and discussion

In this study, the detection of compounds in *Strobilanthes crispus* by GC-MS were presented in Table 1 to 4. The GC-MS analysis of methanol leaf extract revealed the presence 18 main compounds with

9,12,15-Octadecatrienoic acid, (Z,Z,Z)- present in the most (26.21%), followed by squalene (26.11%), stigmasterol (10.93%), vitamin E (9.75%), gamma-sitosterol (6.70%), campesterol (3.57%) and others. The detected compounds reported in this study was similar with Muslim *et al.* (2010) but was lesser as only single quadruple (MS) technology was used in this study.

Meanwhile, GC-MS analysis of methanol stem extract revealed the presence 23 main compounds with lupeol (25.58%) present in the most, followed by 9,12-octadecadienoic acid (Z,Z)- (14.41%), 9,12-octadecadienoic acid (Z,Z)- (13.23%), gamma.-sitosterol (8.47%), campesterol (4.85%), vitamin E (3.18%), betulin (2.38%), squalene (1.63%) and others. On the other hand, the GC-MS analysis of methanol root extract revealed the presence of only 7 main compounds with beta.-amyrin (49.26%) present in the most, followed by heneicosane (5.55%), stigmasterol (5.30%), squalene (1.04%) and others. Besides, the GC-MS analysis of methanol callus extract revealed the presence 12 main compounds in the tissue culture, with the presence of beta.-humulene (26.22) at the most, 4,22-Stigmastadiene-3-one (9.08%), Stigmast-4-en-3-one(5.56%), stigmasterol (1.86%) and others.

Overall, the *S. crispus* plant was found to rich with terpenoid such as squalene, which was present in all the leaf, stem and root, but not in the callus. Squalene is an important precursor for the sysnthesis of phytosterols such as stigmasterols, campesterols, sitosterols, as well as precursor for triterpenoids such as lupeol, amyrin and betulin. Squalene has also been reported to act as chemopreventive agent by Smith (2000). Besides, this plant was also rich with various types of phytosterols such as stigmasterols, campesterols, sitosterols, as well as triterpenoids such as lupeol, amyrin and betulin. All of these compounds have been reported to possess anti-tumor and anti-inflammatory activities (Atif *et al.*, 2003; Ghosh *et al.*, 2011). Interestingly, lupeol and betulin were only present in the stem part, but not the other parts of this plant.

Besides, the callus induced from this plant was found to contain bioactive compounds such as humulene and stigmasterol with also its derivatives which were not found in other parts of the plant. Humulene has been found to produce anti-inflammatory effects in mammals, and has potential to be a tool in the management of inflammatory diseases. Meanwhile, stigmasterol has been reported to induce apoptosis in Ehrlich's ascites carcinoma in mice through the activation of protein phosphatase 2A via ceramide (Ghosh *et al.*, 2011).

Table 1. GC-MS analysis revealed the presence of phytochemicals in methanol leaf extract of *S. crispus*.

No.	Retention Time (R/T), Minute	Name of the Compound	Molecular Formula	MW	Peak Area %
1	4.311	13-Tetradece-11-yn-1-ol	$C_{14}H_{24}O$	208	0.46
2	4.696	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	26.21
3	8.391	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	0.01
4	11.900	6-Tetradecanesulfonic acid, butyl ester	$C_{18}H_{38}O_3S$	335	0.24
5	12.145	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	331	1.05
6	12.343	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	331	0.40
7	14.231	Eicosane	$C_{20}H_{42}$	283	0.30
8	16.102	Cyclododecyne	$C_{12}H_{20}$	164	1.05
9	18.649	Squalene	$C_{30}H_{50}$	411	26.11
10	21.558	2-Methyl-3-(3-methyl-but-2-enyl)-2 -(4-methyl-pent-3-enyl)-oxetane	$C_{15}H_{26}$	222	0.17
11	22.036	gamma.-Tocopherol	$C_{28}H_{48}O_2$	417	0.34
12	22.852	Cholesterol	$C_{27}H_{46}O$	387	1.96
13	23.225	Vitamin E	C29H50O2	431	9.75
14	24.367	Campesterol	$C_{28}H_{48}O$	401	3.57
15	24.833	Stigmasterol	$C_{29}H_{48}O$	413	10.93
16	25.580	gamma.-Sitosterol	C29H50O	415	6.70
17	25.947	beta.-Amyrin	$C_{30}H_{50}O$	427	1.47
18	27.200	Vitamin E	C29H50O2	431	0.25

Table 2. GC-MS analysis revealed the presence of phytochemicals in methanol stem extract of *S. crispus*.

No.	Retention Time (R/T), Minute	Name of the Compound	Molecular Formula	MW	Peak Area %
1	4.317	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294	0.90
2	4.515	10-Heneicosene (c,t)	$C_{21}H_{42}$	295	2.45
3	4.713	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	14.01
4	5.395	Eicosane	$C_{20}H_{42}$	283	2.36
5	7.039	Hexadecane	$C_{16}H_{34}$	226	0.98
6	7.120	Nonadecane	$C_{19}H_{40}$	269	0.89
7	8.449	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	0.10
8	9.236	Tetracosane	$C_{24}H_{50}$	339	0.95
9	9.283	Pentadecane	$C_{15}H_{32}$	212	2.98
10	11.941	Nonadecane	$C_{19}H_{40}$	269	1.09
11	13.106	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	391	0.18
12	16.091	1,3,12-Nonadecatriene	$C_{19}H_{34}$	262	0.63
13	18.591	Squalene	$C_{30}H_{50}$	411	1.63
14	20.491	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	$C_{13}H_{10}F_3N_3O$	281	0.14
15	22.042	gamma.-Tocopherol	$C_{28}H_{48}O_2$	417	0.19
16	22.852	Cholesterol	$C_{27}H_{46}O$	387	1.49
17	23.213	Vitamin E	C29H50O2	431	3.18
18	24.367	Campesterol	$C_{28}H_{48}O$	401	4.85
19	24.851	Stigmasterol	$C_{29}H_{48}O$	413	13.23
20	25.591	gamma.-Sitosterol	C29H50O	415	8.47
21	25.953	beta.-Amyrin	$C_{30}H_{50}O$	427	0.64
22	26.635	Lupeol	$C_{30}H_{50}O$	427	25.58
23	32.329	Betulin	$C_{30}H_{50}O_2$	443	2.38

Table 3. GC-MS analysis revealed the presence of phytochemicals in methanol root extract of *S. crispus*

No.	Retention Time (R/T), Minute	Name of the Compound	Molecular Formula	MW	Peak Area %
1	7.007	Eicosane	$C_{20}H_{42}$	283	2.87
2	7.071	Heneicosane	$C_{21}H_{44}$	297	5.55
3	8.476	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	1.75
4	13.119	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	391	0.16
5	18.561	Squalene	$C_{30}H_{50}$	411	1.04
6	24.749	Stigmasterol	$C_{29}H_{48}O$	413	5.30
7	26.506	beta.-Amyrin	$C_{30}H_{50}O$	427	49.26

Table 4. GC-MS analysis revealed the presence of phytochemicals in methanol root extract of *S. crispus*

No.	Retention Time (R/T), Minute	Name of the Compound	Molecular Formula	MW	Peak Area %
1	3.594	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.49
2	5.855	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	4.51
3	6.106	2-Methyl-Z,Z-3,13-octadecadienol	$C_{19}H_{36}$	280	2.03
4	17.839	cis,cis,cis-7,10,13-Hexadecatriena	$C_{16}H_{26}$	234	1.82
5	19.285	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	0.93
6	26.390	Stigmasterol	$C_{29}H_{48}O$	413	1.86
7	27.211	beta.-Sitosterol	C29H50O	415	0.61
8	27.357	Stigmastanol	C29H52O	417	0.34
9	27.742	alpha.-Amyrin	$C_{30}H_{50}O$	427	0.35
10	28.430	4,22-Stigmastadiene-3-one	$C_{29}H_{46}O$	411	9.08
11	28.529	beta.-Humulene	$C_{15}H_{24}$	204	26.22
12	29.386	Stigmast-4-en-3-one	$C_{29}H_{48}O$	413	5.56

Conclusion

In conclusion, this study has managed to report more bioactive compounds that was not reported for *S. crispus* before as the study covered the analysis of leaf, stem, root and also callus of the plant. Callus was found to accumulate bioactive compound such as humulene and stigmasterol with its derivatives which were not found in other parts of the plant. Further study such as isolation and bioassay of these derivatives should be carried out in the future.

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