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GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF PHYTOCHEMICALS OF FIRST GENERATION GAMMA-IRRADIATED TYPHONIUM FLAGELLIFORME LODD. MUTANTS

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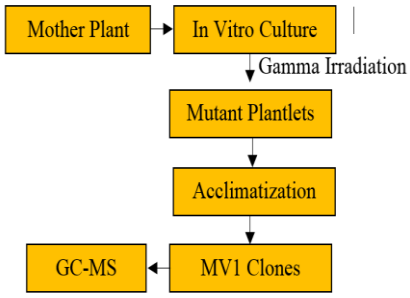
Graphical abstract



Abstract

Rodent tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian herbal plant from *Araceae* family which is highly potential against several types of cancer. However, anticancer compound of rodent tuber is currently unknown. Rodent tuber has a low genetic diversity due to conventional vegetative propagation. In vitro propagation combined with gamma-irradiation of rodent tuber's calli had been performed to increase the genetic diversity of rodent tuber. The mutant plants had been acclimatised and analysed with RAPD molecular markers, but the phytochemical constituents of these mutants have never been investigated. This research utilised gas chromatography-mass spectrometry (GC-MS) analytical method to identify and measure the relative abundances of major phytochemical constituents of rodent tuber control and mutant plants. GC-MS analysis successfully showed phytochemical constituents of the ethanol extract of rodent tuber plants. Shoots and tubers of mutant clones had at least 8 anticancer compounds whose quantities were higher than control plants. Shoots and tubers of mutant clones also contained new anticancer compounds which were not found in control plants. Shoots of mutant clones contained new anticancer compounds such as 7-pentadecyne, β-sitosterol, hexadecanoic acid methyl ester, cis-vaccenic acid, ergost-5-en-3-ol (campesterol). Tubers of mutant clones contained new anticancer compounds such as β-sitosterol, ethyl palmitate, hexadecanoic acid ethyl ester, vitamin E (alpha tocopherol), ergost-5-en-3-ol (campesterol). Rodent tuber mutant clones are therefore very potential to be developed as anticancer drugs.

Keywords: Typhonium flagelliforme Lodd., mutant, GC-MS,phytochemical

1.0 INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian herbal plant from *Araceae* family [1]. Ethanol fraction of rodent tuber extract had been proven to be effective in inhibiting proliferation of breast cancer cells T47D [2]. Dichloromethane fraction of rodent tuber's tuber was able to inhibit MCF-7 breast cancer cells [3], human T4-lymphoblastoid cancer cells [4, 5], and NCI-H23 non-small lung carcinoma cells [6]. Phytochemical constituents of rodent tuber are alkaloid, steroid, flavonoid and glycoside [1]. Rodent tuber also had toxicity against *Artemia salina* [7], and was able to induce apoptosis of cancer cells [6]. Anticancer compound of rodent tuber is currently unknown.

Rodent tuber has a low genetic diversity due to conventional vegetative propagation method [8]. The development of rodent tuber as anticancer drugs faced obstacles due to its low genetic diversity [9]. Genetic diversity is tightly related to the composition of anticancer bioactive compounds which is a key factor for drug formulation. Anticancer compounds of rodent tuber can be increased by inducing mutation of in vitro calli or shoot explants. Sianipar *et al*. (2011) [10] had induced, proliferated, and regenerated in vitro calli (somatic cell populations) of rodent tuber through single node culture method.

Gamma irradiation is a physical mutagen commonly used to induce mutation of plants. Sianipar *et al*. (2013b) [11] had produced in vitro putative mutant clones of rodent tuber by gamma irradiation at LD_{50} dose of 25 Gy. Sianipar *et al*. (2013c) [12] also had produced rodent tuber mutant clones by irradiating somatic cell populations with gamma ray at the dose of 6 Gy. First generation in vitro mutants had been detected by RAPD molecular marker and showed genetic differences with control (Sianipar *et al*., 2015a) [13]. Rodent tuber plantlets were then acclimatizated in green house and produced 37 clones of first generations of putative mutants (MV1) which had a higher amount of biomass if compared to control (Sianipar *et al*., 2013c) [12]. MV1 had shown genetic changes from control based on RAPD molecular marker analysis (Sianipar *et al*., 2015b) [14].

Careful investigation of phytochemical constituents of MV1 has not been performed before. Information of phytochemical constituents are important for discovery of novel therapeutics and development of semi-synthetic and synthetic compounds [15]. Gas chromatography-mass spectrometry (GC-MS) is an analytical method to separate compounds with gas moving phase and identify their molecular weights [16]. GC-MS had been successfully used to analyse phytoconstituents and bioactive compounds of herbal plants *Melia orientalis* [17] and *Maranta arudinacea* L. [18]. Chemical composition of the essential oil from javanian Pimpinella pruatjan Molk [19].GC-MS analysis had also been performed in non-polar active fraction of Malaysian rodent tuber [20]. This research aimed to analyse the chemical constituents of ethanol extract of first generation gamma-irradiated rodent tuber putative mutants (MV1) with GC-MS.

2.0 METHODOLOGY

2.1 Plant Material and Extract Preparation

Rodent tuber plant was isolated, propagated in vitro (Sianipar *et al*., 2011) [10], and treated with gamma-irradiation to induce genetic mutation (Sianipar *et al*., 2013b) [11]. Rodent tuber plantlets were acclimatised (Sianipar *et al*., 2015b) [14]. Mutant and control plants were harvested for extraction.

A control plant and three first generation putative mutant clones (MV1), i.e. 6-3-3-6, 6-1-1-1, 6-2-5-2 were used in extraction and GC-MS analysis. Shoots and tubers of the plants were dried and homogenised. Homogenised samples were macerated in 100ml of ethanol 96% overnight for 2 nights. Ethanol extracts were filtered with Whatman filter paper.

2.2 GC-MS Analysis

Ethanol extracts were injected into the column with conditions: 5µl injection volume with 5:1 split ratio and 250°C injection temperature. Helium was used as carrier gas with velocity 0.8µl/minute. Column temperature was set at 70°C with 5°C/minute increment. When temperature reached 200°C, the temperature remained constant for 1 minute and then further increased at 20°C/minute increment until the temperature reached 280°C. The temperature remained constant for another 28 minutes. Mass spectrometer was operated in electron impact ionisation mode with 70 eV voltage.

2.3 Identification of Phytocomponents

Identification of GC-MS mass spectrum was performed using the National Institute Standard Technique (NIST) database with ≥90% fit factor. The relative abundance percentage of each compound was calculated by comparison of its average peak area to the total area.

3.0 RESULTS AND DISCUSSION

GC-MS analysis successfully showed major phytochemical constituents of the shoot (Table 1) and tuber (Table 2) of control rodent tuber plant. Fifteen compounds were identified in the ethanol extract of the shoot of control. Five compounds with highest relative abundance were cis-13-octadecenoic acid (29.69%), oleic acid (25.98%), palmitic acid (16.03%), 14-methyl-8-hexadecyn-1-ol (3.45%) and neophytadiene (3.22%). Other compounds constituted 21.63% of the extract. The

total percentage of the unidentified compounds was 0.00%.

Twelve compounds were identified in the ethanol extract of the tuber of control rodent tuber plant. Five compounds with the highest relative abundance were cis-13-octadecenoic acid (38.00%), palmitic acid (25.20%), stearate (20.78%), cis,cis-linoleic acid (Grapeseed oil) (3.35%), stigmasterol (2.50%). Other compounds constituted 44.78% of the extract. The total percentage of the unidentified compounds was 3.39%.

GC-MS analysis showed different relative abundance of major phytochemical constituents in the shoot and tuber of rodent tuber control plant. Several compounds that were produced in higher abundance in the shoot were 3,7,11,15-tetramethyl-2-hexadecene, neophytadiene, ethyl palmitate, methyl elaidate, 2-hydroxycyclopentadecanone, phytol isomer, 9-octadecenoic acid, oleic acid, and vitamin E (alpha tocopherol). Several compounds that were produced in higher abundance in the tuber were 9,17-octadecadienal, stearate (octadecanoic acid), Grapeseed oil (cis,cis-linoleic acid), n-heptacosane, n-nonacosane, n-octacosane, and delta-5-ergostenol.

Table 1 Phytochemical constituents of the shoot of control rodent tuber plant based on GC-MS analysis

Retention Time	Compound Name[a]	Relative Abundance (%)[b]
21.745	3,7,11,15-tetramethyl-2-hexadecene	0.93
21.808	neophytadiene	3.22
21.849	3,7,11,15-tetramethyl-2-hexadecene	2.55
22.014	neophytadiene	1.03
22.166	3,7,11,15-tetramethyl-2-hexadecene	1.48
22.980	ethyl palmitate	0.63
23.083	palmitic acid	16.03
23.628	methyl elaidate	0.88
23.649	2-hydroxycyclopentadecanone	0.59
23.738	phytol isomer	7.56
23.966	9-octadecenoic acid	0.78
24.097	cis-13-octadecenoic acid	29.69
24.145	oleic acid	25.98
24.635	14-methyl-8-hexadecyn-1-ol	3.45
25.510	2-hydroxycyclopentadecanone	1.92
27.593	Spinacene (trans squalene)	1.51
31.282	vitamin E (alpha tocopherol)	1.52
33.640	stigmasterol	0.25

[a]Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥90%. [b]Relative abundance was determined based on area percentage of each compound.

Tabel 2 Phytochemical constituents of the tuber of control rodent tuber plant based on GC-MS analysis

Retention Time	Compound Name[a]	Relative Abundance (%)[b]
23.104	palmitic acid (hexadecanoic acid)	25.20
23.621	palmitic acid (hexadecanoic acid)	1.17
23.959	9,17-octadecadienal	0.83
24.118	cis-13-octadecenoic acid	38.78
24.173	stearate (octadecanoic acid)	20.78
24.628	Grapeseed oil (cis,cis-linoleic acid)	3.35
25.497	14-methyl-8-hexadecyn-1-ol	0.72
26.600	n-heptacosane	0.26
27.586	Spinacene (trans squalene)	0.48
28.082	n-nonacosane	1.43
30.227	n-octacosane	0.85
33.068	delta-5-ergostenol	1.43
33.619	Stigmasterol	2.50

[a]Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥90%. [b]Relative abundance was determined based on area percentage of each compound.

Several compounds analysed in the control rodent tuber had important biological activities for medical application. Palmitic acid had been shown to have antitumour activity in mice model and leukaemia cells [21]. Spinacene is potent for nucleic acid-based drug discovery [22]. Vitamin E is a potent antioxidant [23]. Antiproliferative effect of stigmasterol had been shown against prostate cancer cell PC3 through apoptotic induction mechanism [24]. Stearate has antiviral and anti-inflammatory effect [25].

GC-MS analysis successfully identified 21 major compounds in ethanol extract of mutant rodent tuber 6-1-1-1 shoot (Figure 1, Table 3). Five major compounds with highest relative abundance were cis-13-octadecenoic acid (25.26%), phytol (11.25%), cis-vaccenic acid (10.84%), palmitic acid (9.60%), and 5-chloro-6nitrocholestan-3-yl acetate (6.18%). Other compounds constituted 26.09% of the extract. The total percentage of unidentified compounds was 10.78%.

Several compounds that were detected in the shoots of mutant clone 6-1-1-1 and not in control plant were 2-hexadecen-1-ol, 3,7,11,15 - tetramethyl, methyl-11-octadecenoate, cis-vaccenic acid, 7-pentadecyne, icosane, 11-tricosene, hexacosene, ergost-5-en-3-ol, beta-sitosterol, 5-chloro-6nitrocholestan-3-yl acetate, and N'-hydroxy-N-[2-(trifluoromethyl)phenyl]-3-pyridinecarboximidamide. This difference reflected metabolic profile difference between mutant and control plant that was likely due to gamma irradiation of mutant plant.

Tabel 3 Phytochemical constituents of the shoot of mutant 6-1-1-1 rodent tuber plant based on GC-MS analysis

Retention Time	Compound Name[a]	Relative Abundance (%)[b]
21.746	3,7,11,15-tetramethyl-2-hexadecene	0.78
21.815	neophytadiene	4.56
21.856	3,7,11,15-tetramethyl-2-hexadecene	2.14
22.015	2-hexadecen-1-ol, 3,7,11,15-tetramethyl	1.27
22.166	2-hexadecen-1-ol, 3,7,11,15-tetramethyl	1.93
23.125	Palmitic acid (hexadecanoic acid)	9.60
23.628	methyl-11-octadecenoate	0.52
23.656	methyl-11-octadecenoate	0.37
23.759	phytol	11.25
23.973	9-octadecenoic acid	0.25
24.159	cis-13-octadecenoic acid	25.26
24.207	cis-vaccenic acid	10.84
24.649	7-pentadecyne	0.56
27.620	squalene	1.95
28.103	icosane	0.22
28.269	11-tricosene	0.54
30.537	hexacosene	0.81
31.330	vitamin E	1.88
33.150	ergost-5-en-3-ol (campesterol)	1.59
33.723	stigmasterol	2.09
34.874	beta-sitosterol	0.89
40.846	5-chloro-6nitrocholestan-3-yl acetate	6.18
48.789	N'-hydroxy-N-[2-(trifluoromethyl)phenyl]-3-pyridinecarboximidamide	3.74

[a]Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥90%. [b]Relative abundance was determined based on area percentage of each compound.

GC-MS analysis showed 19 major compounds in tuber of mutant rodent tuber 6-1-1-1 (Figure 1, Table 4). Five major compounds with the higest relative abundance were cis,cis-linoleic acid (Grapeseed oil) (53.5%), palmitic acid (27.73%), stearate (octadecanoic acid) (6.33%), ethyl-9,12-octadecadienoate (1.73%), and 9,12-octadecanoic acid (1.42%). Other compounds constituted 9,17% of the extract. The total percentage of the unidentified compounds was 0.12%.

Tabel 4 Phytochemical constituents of the tuber of mutant 6-1-1-1 rodent tuber plant based on GC-MS analysis

Retention Time	Compound Name[a]	Relative Abundance (%)[b]
21.628	Myriistic acid	0.11
22.973	palmitic acid ethyl ester	1.08
23.200	palmitic acid (hexadecanoic acid)	27.73
23.545	palmitic acid	0,40
23.952	ethyl-9,12-octadecadienoate	1,73
23.993	ethyl linolenate	0.48
24.221	Grapeseed oil (cis,cis-linoleic acid)	48.28
24.269	stearate (octadecanoic acid)	6.33
24.352	Grapeseed oil (cis,cis-linoleic acid)	3.07
24.510	9,12-octadecanoic acid	1.42
24.621	Grapeseed oil (cis,cis-linoleic acid)	2.15
24.993	cyclopentadecanone	0.72
25.076	2-aminoethanethiosulphonic acid	0,62
25.503	3-dodecyl-2,5-furandione	0,35
26.607	n-heptacosane	0,35
27.600	Spinacen (trans squalene)	0,73
28.103	n-nonacosane (celidoniol)	1.03
30.247	Docosane	0.58
31.268	vitamin E	0.37
33.102	Campesterol	0.75
33.661	β-sitosterol	0.95
34.833	α-dihidrofucosterol	0.65

[a]Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥90%. [b]Relative abundance was determined based on area percentage of each compound.

Several major compounds were detected only in the tuber of mutant rodent tuber 6-1-1-1, not in tuber of control rodent tuber. These compounds were myriistic acid, palmitic acid ethyl ester, ethyl-9,12-octadecadienoate, ethyl linolenate, 9,12-octadecanoic acid, cyclopentadecanone, 2-aminoethanethiosulphonic acid, 3-dodecyl-2,5-furandione, docosane, campesterol, beta-stigmasterol (BSS), and alpha dihidrofucosterol.

In addition to palmitic acid, squalene, vitamin E, stigmasterol and stearate, several other compounds with important biological activity for medical applications were found in the tuber of mutant rodent tuber plant. Pythol could lower triglyceride and cholesterol content in blood [26]. Cis-vaccenic acid could be used in the prediction of *chronic kidney disease* (CKD) [27]. 7-pentadecyne's structure resembles the activator of protein kinase C, making it a potent anticancer agent [28]. β-sitosterol (BSS) has anti-inflamatory, antineoplastic, antipiretic, and immunomodulatory effects [29].

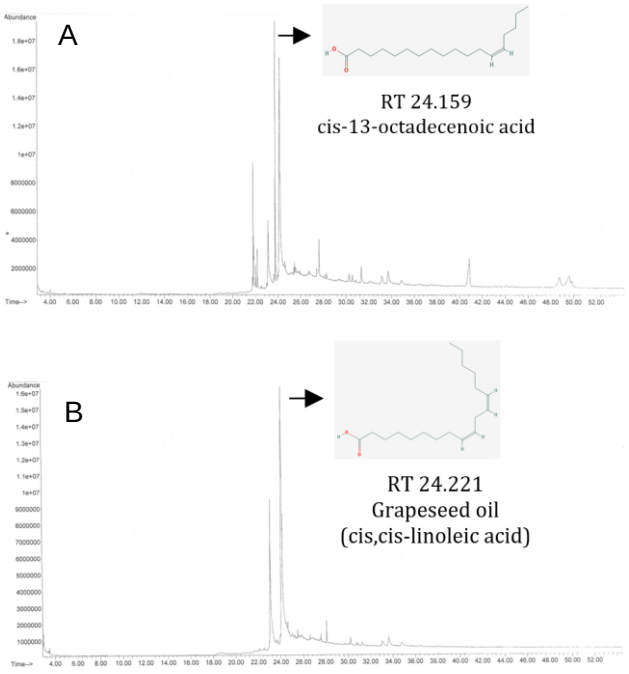


Figure 1 GC-MS chromatogram of mutant clone 6-1-1-1 a) shoot of 6-1-1-1; b) tuber of 6-1-1-1. X-axis represents retention time while Y-axis represents relative abundance. The chemical structure of compounds with the highest relative abundances were shown (chemical structure obtained from PubChem, NCBI)

Figure 2 highlighted the difference of relative abundances of anticancer compounds between shoot and tuber of control and three mutant rodent tuber clones, i.e. 6-1-1-1, 6-3-3-6 and 6-2-5-2. The quantities of several anticancer compounds in mutant clones were higher than control. Shoots and tubers of mutant clones had at least 8 anticancer compounds whose quantities were higher than control. Shoots and tubers of mutant clones also contained new anticancer compounds which were not found in control plants. Shoots of mutant clones contained new anticancer compounds such as 7-pentadecyne, β-sitosterol, hexadecanoic acid methyl ester, cis-vaccenic acid, ergost-5-en-3-ol (campesterol). Tubers of mutant clones contained new anticancer compounds such as β-sitosterol, ethyl palmitate, hexadecanoic acid ethyl ester, vitamin E (alpha tocopherol), ergost-5-en-3-ol (campesterol). These differences reflected the effect of gamma irradiation to metabolic profile of mutants in comparison to wildtype control plant.

Compound Name	Relative Abundance (%)							
	Shoot				Tuber			
	Control	6-3-3-6	6-1-1-1	6-2-5-2	Control	6-3-3-6	6-1-1-1	6-2-5-2
Stigmasterol [23, 29]	0.25	1.94	2.09	2.20	2.50	1.38	0.95	1.33
7-pentadecyne [27]	NA	1.39	0.56	0.61	NA	NA	NA	NA
β-sitosterol [28, 30]	NA	NA	0.89	NA	NA	NA	0.95	NA
Grapeseed Oil (Cis,cis-linoleic acid) [19]	NA	NA	NA	NA	3.35	NA	48.28	42.97
Ethyl palmitate [6]	0.63	NA	NA	NA	NA	0.64	NA	0.67
Palmitic acid (hexadecanoic acid) [6, 20]	16,03	7,97	9,60	11,61	26.37	21,38	28.13	23.55
Hexadecanoic acid methyl ester [31]	NA	0.44	NA	NA	NA	NA	NA	NA
Hexadecanoic acid ethyl ester [32]	NA	NA	NA	NA	NA	NA	1.08	NA
Spinacene (squalene) [33]	1,51	2,17	1,95	1,87	0,48	NA	0,73	0,43
Vitamin E (alpha tocopherol) [34, 35]	1,52	1,54	1,88	2,56	NA	NA	0,37	0,46
cis-vaccenic acid [36]	NA	26,54	10,84	15,23	NA	NA	NA	NA
ergost-5-en-3-ol (campesterol) [37, 38, 39, 40]	NA	1,16	1,59	NA	NA	1,18	0,75	0,82

Figure 2 Comparison of the Relative Abundances of Anticancer Compounds in Shoot and Tuber of Control and Mutant Rodent Tuber Plant based on GC-MS analysis. NA is not available, compound amount was not high enough to be detected by GC-MS relative to other compounds in the sample. The quantities of anticancer bioactive compounds of MV3 putative mutant clones which were higher than control were indicated by the yellow highlights. References which stated that those compounds have anticancer activity were indicated in brackets"[]"

Following this research, several researches can be performed to develop rodent tuber as the source of anticancer medicine. Similar investigation of phytochemical constituents of next generations mutant rodent tuber should be performed to ensure consistent metabolite profile. Another important research that should be carried out is the in vitro and in vivo anticancer activity assays of rodent tuber against several cancer cell lines.

4.0 CONCLUSION

GC-MS analysis successfully showed phytochemical constituents of the ethanol extract of rodent tuber control and mutant plants. Similarities and differences in compound's relative abundance were observed in the shoot and tuber of both control and mutant plants. Relative abudances of several anticancer compounds were higher in mutant clones compared to control plants. Mutant clones also contained new anticancer compounds which were not found in control plants. Rodent tuber mutant clones therefore are highly potential to be developed as anticancer drugs. This research was the first to show phytochemical constituents of gamma-irradiated rodent tuber mutant plants.

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