Bioactive compounds of fourth generation gamma-irradiated \emph{Typhoniumflagelliforme} Lodd. mutants based on gas chromatography-mass spectrometry

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Abstract. Rodent tuber (\emph{Typhonium flagelliforme}Lodd.) is an Indonesian anticancer medicinal plant. The natural genetic diversity of rodent tuber is low due to vegetative propagation. Plant’s genetic diversity has to be increased for obtaining clones which contain a high amount of anticancer compounds. \textit{In vitro} calli were irradiated with 6 Gy of gamma ray to produce \textit{in vitro} mutant plantlets. Mutant plantlets were acclimated and propagated in a greenhouse. This research was aimed to identify the chemical compounds in the leaves and tubers of the fourth generation of rodent tuber’s vegetative mutant clones (MV4) and control plants by using GC-MS method. Leaves and tubers of MV4 each contained 2 and 5 anticancer compounds which quantities were higher compared to control plants. MV4 leaves contained 5 new anticancer compounds while its tubers contained 3 new anticancer compounds which were not found in control. The new anticancer compounds in leaves were hexadecanoic acid, stigmast-5-en-3-ol, ergost-5-en-3-ol, farnesol isomer \textit{a}, and oleic acid while the new anticancer compounds in tubers were alpha tocopherol, ergost-5-en-3-ol, and beta-elemene. Rodent tuber mutant clones are very potential to be developed into anticancer drugs.

1. Introduction

Rodent tuber (\emph{Typhonium flagelliforme} Lodd.) is a native Indonesian medicinal plant from Araceae family [1]. Rodent tuber contains antineoplastic/anticancer and antivirus chemical agents[2]. Bioactive compounds in this plant are alkaloids, saponins, steroids, and glycosides [3]. Rodent tuber’s extract was cytotoxic against the cancer of lung, breast [4], liver [5], blood(leukemia)[6], colon, prostate gland, and cervix [7]. Its hexane extract was cytotoxic against \emph{Artemia salina} larvae[8]. It also had antibacterial and antioxidant properties [9].

Rodent tuber from Indonesia has a low genetic diversity because it is usually propagated by conventional vegetative methods[1]. Genetic diversity must be increased in order to obtain plant clones which contain a high amount of anticancer compounds. Mutation induction is one of the ways...
to increase plant’s genetic diversity. Mutation could be induced by irradiating plants with physical mutagens, such as gamma ray.

*In vitro* embryogenic somatic cell population/calli of rodent tuber plant from Bogor have been induced, proliferated, and regenerated by single node culture method [10]. The mutation induction of those embryogenic calli were done by combining the effects of 6 Gy gamma irradiation and *in vitro* somaclonal variation. *In vitro* plantlets, which were regenerated from those induced calli, showed various morphological characters [11] and had genetic differences from control plants according to Randomly Amplified Polymorphic DNA (RAPD) molecular marker analysis [12]. *In vitro* plantlets were acclimated in a greenhouse. There were 37 clones of the first generation of rodent tuber vegetative mutant (MV1). MV1 clones had various morphological characters [13]. Out of those 37 MV1 clones, there were 17 clones which were genetically different from control plants according to RAPD [14].

Genetic mutation may result in a change of the composition and quantities of phytochemical compounds in a plant, which could be detected by Gas Chromatography-Mass Spectrometry (GC-MS). GC uses gas as the mobile phase to separate chemical compounds. MS will identify those separated compounds by referring to a database [15]. GC-MS has been used to identify the chemical contents of herbal plants, such as *Meliaorientalis* [16], *Maranta arudinacea* L. [17], and the nonpolar fraction of the Malaysian rodent tuber [18].

MV1 clones of rodent tuber have been propagated and regenerated into the fourth generation mutant clones (MV4). The purpose of this research was to identify the chemical composition and analyzing the anticancer bioactive components of the polar fraction of leaves and tubers of rodent tuber MV4 clones by using GC-MS method.

2. Materials and methods

2.1. Plant materials and extract preparation

Rodent tuber plant from Bogor, Indonesia was isolated, propagated *in vitro* [10], and irradiated with gamma ray to induce genetic mutation [11]. Rodent tuber plantlets were acclimated in a greenhouse [13]. Mutant and control plants were harvested for extraction. One control plant and eight MV4 mutant clones, i.e. 6-9-1, 6-3-3-6, 6-1-1-6, 6-3-2-5, 6-6-3-6, 6-2-8-2, 6-1-1-2, 6-1-2 were analyzed in this research. Shoots and tubers were dried and homogenised. Homogenised samples were macerated in 100ml of ethanol 96% and incubated overnight for two days to obtain chemical components in its ethanol fractions. Ethanol fractions were filtered with Whatman filter paper.

2.2. GC-MS analysis

Sample’s ethanol fractions were injected into the GC column. Injection volume was 5µl with 5:1 split ratio and the injection temperature was 250°C. Helium was used as carrier gas with velocity 0.8 µl per minute. Column temperature was set at 70°C with 5°C per minute increment. When the temperature reached 200°C, it remained constant for 1 minute and then will be increased at the rate of 20°C per minute 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 phytochemical compounds

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

3. Results and discussion

GC-MS has successfully identified phytochemicals in the leaves and tubers of rodent tuber’s control and MV4 clones (Figure 1). There were 32 different chemical compounds in theethanolic fraction of a control plant. The 5 most abundant compounds were neophytadiene (10.6%), phytol isomer (13.07%),

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alpha tocopherol (8.85%), campesterol (7.23%), and stigmasterol (8.90%). Ethanolic fractions of the tubers contained at least 33 different chemical compounds, the most abundant were 9,12-octadecadienoic acid (7.67%), octacosane (9.35%), nonacosane (11.03%), octacosane (8.66%), and stigmasterol (7.88%).

Figure 1. A: control; B: MV4 clone 6-1-2; C: MV4 clone 6-3-2-5; D: MV4 clone 6-1-1-6. Rodent tuber control and mutant clones after 20 weeks of growing in a green house.

GC-MS analysis revealed that leaves and tubers of control plants contained a range of different phytochemicals. The phytochemicals which were contained in higher amounts in leaves than in tubers were phytol, squalene, and stigmasterol. Whilst, tubers contained higher amounts of hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester, hexadecanoic acid, 9,12-octadecadienoic acid methyl ester, 9,12,15-octadecatrienoic acid methyl ester, 9,12-octadecadienoic acid ethyl ester, hexadecanamide, 4,22-stigmastadiene-3-one, and stigmast-4-en-3-one compared to leaves.

Based on GC-MS analysis, there were chemical composition differences between control and mutant clones and between each of the different clones of mutant plants. Some anticancer bioactive compounds were present in higher amounts in the mutant clones compared to in control plants. Mutant clones also contained several new anticancer compounds which were not found in control (figure 2, table 1 and 2). Leaves of mutant clones contained higher amounts of two anticancer compounds compared to control, i.e. hexadecanoic acid ethyl ester and hexadecanoic acid methyl ester. They also contained five new anticancer compounds, i.e. hexadecanoic acid, stigmaster-5-en-3-ol, ergost-5-en-3-ol, farnesol isomer a, and oleic acid. Tubers of mutant clones contained higher amounts of five anticancer compounds, i.e. hexadecanoic acid, hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester, squalene, and 2-methoxy-4-vinylphenol. Among those five compounds, the relative abundance of hexadecanoic acid in 6-6-3-6 clone was the most different compared to control, i.e. increasing 30.63% from control plants.

The higher amounts of some anticancer compounds in the mutant compared to control plants has also been observed in GC-MS profile of rodent tuber first generation mutant clones (MV1) [19]. Leaves and tubers of MV1 contained higher amounts of three and four anticancer compounds compared to control, respectively. In addition, leaves and tubers of MV1 each contained four new anticancer compounds which were not found in control plants.

Hexadecanoic acid (palmitic acid) was selectively cytotoxic against leukemia cancer cells MOLT-4 due to its interaction with DNA topoisomerase I and its ability to induce apoptosis. Hexadecanoic acid had in vivo antitumor activity[20]. Biological activities of hexadecanoic acid ethyl ester were antioxidant, antimicrobial[21], and could reduce the risk of coronary heart disease. Hexadecanoic acid methyl ester was able to inhibit the growth and induce apoptosis of human gastric cancer cells[22]. Stigmast-5-en-3-ol (3.beta.,24s) (beta-sitosterol) is a phytosterol with various biological activities. It could reduce the cell’s cholesterol level, modify membrane lipid profile [23], and it was antidiabetic [24], and able to inhibit the growth of cancer cells. Ergost-5-en-3-ol (3 beta) (campesterol) is a phytosterol which has been proven to be able to inhibit various cancer cells, such as lung [25], gastric [26], and ovary [27] cancers. Farnesol is a non-sterol isoprenoid which is commonly found in various fruits and aromatic plants, such as citrus, sage, spearmint, nutmeg, basil, lemon grass, and chamomile. Farnesol could selectively inhibit the proliferation and induce apoptosis of leukemia and cervical cancer cells[28]; [29]. Farnesol had in vivo antitumor and anticarcinogenic activities [30]; [31].
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Table 1. Comparison of the composition of anticancer compounds in leaves of rodent tuber control and MV4 mutant clones based on GC-MS. NA is not available, which means that the quantity of a compound was not high enough to be detected by GC-MS. The quantities of anticancer bioactive compounds in MV4 mutant clones which were higher than control were indicated by the yellow highlights.

| Name of Compound                      | Relative abundance (%) in control | Relative abundance (%) in mutant       |
|---------------------------------------|-----------------------------------|----------------------------------------|
|                                       | 6-9-1 | 6-3-6 | 6-1-6 | 6-3-2-5 | 6-6-3-6 | 6-2-8-2 | 6-1-1-2 | 6-1-2 |
| Hexadecanoic acid, methyl ester       | 39.0  | 1.33  | 0.82  | 0.82    | 0.84    | 0.71    | 0.32    | 0.41   | 0.48  |
| Hexadecanoic acid, ethyl ester       | 0.50  | 0.51  | 0.86  | 0.64    | 1.07    | 1.00    | 0.86    | 0.86   | 1.01  |
| Hexadecanoic acid                    | NA    | 16.7  | 20.89 | 19.46   | 29.09   | 19.33   | 24.53   | 19.36  | 32.85 |
| Stigmast-5-en-3-ol                   | NA    | 3.64  | NA    | NA      | NA      | NA      | NA      | NA     | 2.14  |
| Ergost-5-en-3-ol                     | NA    | NA    | 3.17  | 3.40    | NA      | 2.29    | NA      | 2.47   | NA    |
| Farnesol isomer a                    | NA    | NA    | NA    | NA      | 0.33    | NA      | NA      | NA     | NA    |
| Oleic acid                           | NA    | NA    | NA    | NA      | NA      | 2.03    | NA      | NA     | NA    |

Table 2. Comparison of the composition of anticancer compounds in tubers of rodent tuber control and MV4 mutant clones based on GC-MS. NA is not available, which means that the quantity of a compound was not high enough to be detected by GC-MS. The quantities of anticancer bioactive compounds in MV4 mutant clones which were higher than control were indicated by the yellow highlights.

| Name of Compound                      | Relative abundance (%) in control | Relative abundance (%) in mutant       |
|---------------------------------------|-----------------------------------|----------------------------------------|
|                                       | 6-9-1 | 6-3-6 | 6-1-6 | 6-3-2-5 | 6-6-3-6 | 6-2-8-2 | 6-1-1-2 | 6-1-2 |
| Hexadecanoic acid                    | 7.23  | 19.52 | 26.62 | 10.72   | 27.16   | 37.86   | 32.36   | 36.76  | 25.50 |
| Hexadecanoic acid, methyl ester      | 1.46  | 0.83  | 0.68  | 2.51    | 0.69    | 0.54    | 0.34    | 0.66   | NA    |
| Hexadecanoic acid, ethyl ester       | 0.87  | 1.13  | 1.18  | 2.02    | 1.39    | 1.94    | 1.31    | 1.23   | 3.69  |
| Squalene                             | 0.86  | NA    | NA    | NA      | 1.24    | NA      | 0.49    | 0.55   | 1.32  |
| Alpha tocopherol                     | NA    | 0.24  | NA    | NA      | 0.59    | NA      | NA      | 0.61   | NA    |
| Ergost-5-en-3-ol                     | NA    | 2.95  | 2.85  | 3.31    | 2.43    | 2.56    | 2.20    | 0.36   | 1.35  |
| 2-methoxy-4-vinylphenol              | 0.90  | NA    | NA    | 0.96    | 0.45    | NA      | NA      | 0.46   | NA    |
| Beta-elemene                         | NA    | 4.33  | NA    | 0.13    | NA      | NA      | NA      | NA     | NA    |

Oleic acid is a n-9 monounsaturated fatty acid which was cytotoxic against several types of cancer cells. Oleic acid could inhibit the growth [32] and induce apoptosis [33] of breast cancer cells and inhibit colon adenocarcinoma[34]. Squalene was able to inhibit carcinogenesis of various cancer cells, such as colon cancer [35]. Vitamin E (α and γ-tocopherol) has been proven to be able to reduce the risk of carcinogenesis [36]. 4-vinyl-2-methoxy-phenol was able to inhibit carcinogenesis which is induced by Polycyclic Aromatic Hydrocarbons (PAHs) benzo(a)pyrene (BaP) by regulating the cell cycle protein in order to prevent hyper-phosphorilation of retinoblastome’s tumor suppressor protein [37]. Beta-elemene could induce apoptosis of non-small lung carcinoma by activating caspase-3, -7 and -9, reducing the expression of Bcl-2, inducing the release of cytochrome c, and increasing the level of...
caspase-9 and poly(ADP-ribose) polymerase[38]. Bete-elemene also had antiproliferative activity against prostate carcinoma cell DU145 and PC-3. In addition, it was able to inhibit the growth of lung, colon, cervix, breast, and brain carcinoma cells [39]. Based on GC-MS analysis, mutant clone 6-1-2 had the highest total amount of anticancer compounds in leaves (38.95%) compared to control (0.89%) and the other mutant clones (table 1). GC-MS identified 20 chemical compounds in the ethanolic fraction of 6-1-2’s leaves (Tabel 5). The five most abundant compounds in leaves of 6-1-2 were hexadecanoic acid (32.85%), (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol (8.71%), ethyl (9z,12z)-9,12-octadecadienoate (5.67%), hexadecanoic acid (5.74%), and 9,12-octadecadienoic acid (Z,Z) (16.07%).

![Figure 2](image-url)

**Figure 2.** GC-MS chromatogram of the leaves and tubers of MV4 mutant clones. a) leaves of 6-1-2; b) tubers of 6-6-3-6. X-axis represents retention time while Y-axis represents relative abundance. The chemical structures of compounds with the highest relative abundances were shown (chemical structure was obtained from NIST database).

| Retention Time | Name of Compound\(^a\) | Relative Abundance\(^b\) |
|----------------|-------------------------|-------------------------|
| 31.106         | Neophytadiene           | 1.39                    |
| 31.161         | (2E)-3,7,11,15-tetramethyl-2-hexadecene | 0.81 |
| 31.513         | Neophytadiene           | 0.58                    |

\(^a\)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.
31.906  Hexadecanoic acid, methyl ester  0.48
32.402  Hexadecanoic acid, ethyl ester  1.01
32.657  Hexadecanoic acid  7.03
32.850  Hexadecanoic acid  4.29
32.940  Hexadecanoic acid  2.86
33.057  9,12-octadecadienoic acid (Z,Z)-methyl ester  4.37
33.099  Hexadecanoic acid  3.92
33.209  (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol  8.71
33.430  Ethyl (9z,12z)-9,12-octadecadienoate  5.67
33.471  Hexadecanoic acid  3.49
33.581  Hexadecanoic acid  5.74
33.699  9,12-octadecadienoic acid (Z,Z)  16.07
33.823  Hexadecanoic acid  5.52
34.119  14-methyl-8-hexadecyn-1-ol  4.75
35.181  Methyl 20-methyl-heneicosanoate  0.57
36.312  Tetracosanoic acid, methyl ester  0.33
37.022  Squalene  0.70
39.415  7-bromo-5-(2-bromo-phenyl)-1,3-dihydrobenzo(e)(1,4)diazepin-2-one  3.05
40.125  Stigmastan-3,5-diene  2.68
40.483  Alpha-tocopherol  0.86
42.119  Ergost-5-en-3-ol  2.47
42.759  Stigmasterol  2.96
43.883  Stigmast-5-en-3-ol  2.14
45.455  4,22-stigmastadiene-3-one  1.56

43.39% of compounds identified in the tuber of mutant clone 6-6-3-6 were known to have anticancer activity, which was the highest amount of anticancer compounds compared to control, which anticancer compounds only comprised 11.32% of the identified compounds, and the other mutant clones (table 1). GC-MS identified 18 chemical compounds in the ethanolic fraction of the tuber of MV4 6-6-3-6 clone (table 4). The five most abundant compounds in leaves of MV4 6-6-3-6 clone were hexadecanoic acid (37.86%), 9,12-octadecadienoic acid (Z,Z)-methyl ester (3.39%), ethyl (9z,12z)-9,12-octadecadienoate (8.19%), (9E,12E)-9,12-octadecadienoic acid (25%), and stigmasterol (3.85%).

Table 4. Chemical compounds in tubers of rodent tuber MV4 mutant clone 6-6-3-6 based on GC-MS.

*aCompounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥90%.
*bRelative abundance was determined based on area percentage of each compound.

| Retention Time | Name of Compound | Relative Abundance |
|----------------|------------------|--------------------|
| 31.899         | Hexadecanoic acid, methyl ester | 0.54 |
| 32.409         | Hexadecanoic acid, ethyl ester | 1.94 |
| 32.747         | Hexadecanoic acid | 14.58 |
| 32.837         | Hexadecanoic acid | 3.87 |
| 32.940         | Hexadecanoic acid | 2.50 |
| 33.057         | 9,12-octadecadienoic acid (Z,Z)-methyl ester | 3.39 |
| 33.099         | Hexadecanoic acid | 3.38 |
| 33.202         | Hexadecanoic acid | 3.56 |
| 33.264         | Hexadecanoic acid | 1.33 |
| 33.443         | Ethyl (9z,12z)-9,12-octadecadienoate | 8.19 |
Some chemical compounds, i.e. ethyl (9z,12z)-9,12-octadecadienoate, 14-methyl-8-hexadecyn-1-ol, methyl 20-methyl-heneicosanoate, tetracosanoic acid methyl ester, stigmastan-3,5-diene, and ergost-5-en-3-ol, were detected in leaves of MV4 clone 6-1-2 but not in leaves of control. Tubers of MV4 clone 6-6-3-6 contained seven chemical compounds, i.e. ethyl (9z,12z)-9,12-octadecadienoate, tetracosanoic acid methyl ester, methyl 17-methyl-octadecanoate, 7-bromo-5-(2-bromo-phenyl)-1,3-dihydro-benzo(e)(1,4)diazepin-2-one, ergost-5-en-3-ol, (23s)-ethylcholest-5-en-3beta-ol, and ergost-4-en-3-one,(24R)-, which were not detected in control plants. This finding indicated the difference in metabolomic profile between mutant and control plants due to gamma irradiation-induced genetic mutation.

The difference in chemical contents between control and mutant plants was due to gamma irradiation and somaclonal variation during plant tissue culture process. The application of auxin-type plant growth regulators, such as 2,4-D, in plant culture medium could increase heritable genetic diversity [40]. In addition, the combination of stress condition in in vitro culture and physical mutagen irradiation was able to induce retrotransposon activity, i.e. the movement of DNA from one chromosome to the other. This phenomenon could alter the genetic makeup of a cell, either by increasing or silencing the expression rate of nearby genes or genes which are located next to the retrotransposon [41]. In this respect, the expression of nearby genes which might be responsible for expressing certain organic compounds could also be increased. This research has shown that the usage of plant growth regulators in in vitro culture and the irradiation of calli with gamma ray were able to produce mutant clones which contained a high amount of anticancer compounds. Beside that, gamma irradiation and somaclonal variation were also able to alter the biochemical process [40]. In this research, some MV4 mutant clones showed an increase in the amount of primary and secondary metabolites compared to control plants.

4. Conclusion
The chemical compounds in control and fourth generation vegetative mutant clones (MV4) of rodent tuber have been successfully detected by GC-MS. There were metabolomic profile differences between leaves and tubers, and between mutant and control plants. Leaves and tubers of MV4 each contained 2 and 5 anticancer compounds whose quantities were higher compared to control plants. MV4 leaves contained 5 new anticancer compounds while its tubers contained 3 new anticancer compounds which were not found in control. The new anticancer compounds in leaves were hexadecanoic acid, stigmast-5-en-3-ol, ergost-5-en-3-ol, farnesol isomer α, and oleic acid while the new anticancer compounds in tubers were α-tocopherol, ergost-5-en-3-ol, and β-elemene. The
difference in chemical compounds between mutant and control plants was due to the 6-Gy gamma ray irradiation and somaclonal variation of *in vitro* calli.

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