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ANALYSIS OF BIOACTIVE COMPOUNDS AND MORPHOLOGICAL TRAITS IN INDONESIAN RODENT TUBER MUTANT CLONES OF PEKALONGAN ACCESSION USING GC-MS

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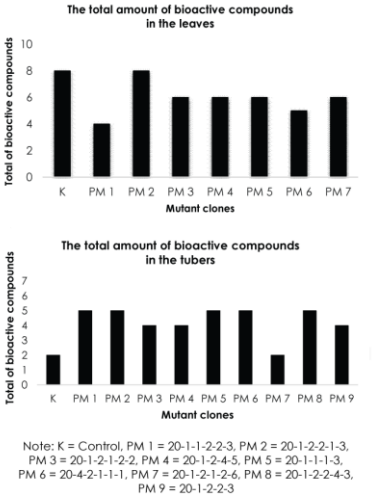
Graphical abstract



Note: K = Control, PM 1 = 20-1-1-2-2-3, PM 2 = 20-1-2-2-1-3, PM 3 = 20-1-2-1-2-2, PM 4 = 20-1-2-4-5, PM 5 = 20-1-1-1-3, PM 6 = 20-4-2-1-1-1, PM 7 = 20-1-2-1-2-6, PM 8 = 20-1-2-2-4-3, PM 9 = 20-1-2-2-3

Abstract

Rodent tuber (*Typhonium flagelliforme* Lodd.) is one of the considerable potential Indonesian medicinal plants. There are many origins of rodent tuber plants in Indonesia such as Pekalongan. Increasing genetic diversity of rodent tuber's Pekalongan accession had been done by using gamma irradiation. Its produced 23 clones of the 7th generation of mutant plants. The focus of this study was on the analysis of biomass and bioactive compounds from those clones using GC-MS. The aim of this study was to determine new bioactive compounds which affect as anticancer in the 7th mutant generation of rodent tuber plant Pekalongan accession. The result showed that 8 mutant clones of rodent tuber had higher biomass than the control. 10 bioactive compounds were detected in the leaves from 7 mutant clones (20-1-1-2-2-3, 20-1-2-2-1-3, 20-1-2-1-2-2, 20-1-2-4-5, 20-1-1-1-3, 20-4-2-1-1-1, 20-1-2-1-2-6) and 7 bioactive compounds in the tubers from 9 mutant clones (20-1-1-2-2-3, 20-1-2-2-1-3, 20-1-2-1-2-2, 20-1-2-4-5, 20-1-1-1-3, 20-4-2-1-1-1, 20-1-2-1-2-6, 20-1-2-2-4-3, 20-1-2-2-3). The bioactive compounds in the mutant clones was 4 times greater than the control. Phytol isomer and eicosane were found as new bioactive compounds in the leaves. 5 new bioactive compounds were found in the tubers for example Hexadecanoic acid ethyl ester, octadecadienoic acid, squalene, beta, eicosane and octacosane. Gamma irradiation is an effective technique to increase the huge numbers of bioactive compounds which can be applied as anticancer, antitumor and antimicobial herbal medicine.

Keywords: *Typhonium flagelliforme* Lodd, Pekalongan accession, gamma ray irradiation, biomass, bioactive compounds

1.0 INTRODUCTION

Treatment uses herbal medicine to be a trend. Herbal medicine has a better compatibility with the human body, lesser side effects and better cultural acceptability. Rodent tuber (*Typonium flagelliforme* Lodd.) is one of the herbal plants with high potential to be developed. In Indonesia, this plant grows throughout the island of Java, in part, of Kalimantan, Sumatra and Papua [15].

The tubers used as a medicine for treating various cancers, including breast cancer [27]. Rodent tuber contains antineoplastic or anticancer and antivirus compounds. Phytochemical analysis showed that Rodent tuber contains alkaloids, saponins, steroids, glycosides, antioxidants [27], phytol, and fatty acids [2]. The active compound was having an

antineoplastic or anticancer that can prevent, kill, and inhibit the growth and spread of cancer cells. Nurrochmad *et al.* [20] demonstrated that the rodent tuber extract had been proven effective to inhibit the growth of breast cancer cells T47D. With various benefits, the rodent tuber is potential to be developed as a raw material of natural medicine.
Rodent tuber also contains bioactive compounds that can inhibit the growth of pathogenic microorganisms such as bacteria, fungi, and virus [20]. The compounds have activity as anticancer and induce apoptosis [4, 27].

Developing of the rodent tuber as a drug faces main problem because the organic compounds are low which is caused by vegetative propagation technique. In plant breeding, in vitro mutagenesis using physical or chemical mutagen can improve the genetic variation, so it is allowing breeders make the selection of genotypes in accordance with the intended purpose. Mutation induction can be done with a mutagen treatment of the plant to be mutated. This technique has been applied to various plants to get new varieties with the desired character. The high genetic variation also affects the diversity and quantity of bioactive compounds Taheri *et al.* [30]. The physical mutation could induce variation in plant morphology [25] and tuber shape [33].

The aim of this study was to determine new bioactive compounds which affect as anticancer, antitumor and antimicobial in the 7^{th} mutant generation of rodent tuber plant Pekalongan accession.

2.0 METHODOLOGY

2.1 Plant Material

The plant material was used rodent tuber Pekalongan accession. The non-irradiated plant was used as a control. There were 23 mutant clones of rodent tuber at 7^{th} generation conducted from Pekalongan accession. Those plants were plantlet in the green house on February – July 2016. The harvesting was done when the plants are 3-4 months old after planting.

2.2 Gamma Irradiation

The plant material irradiation was treated with 6 doses of gamma rays (0, 10, 20, 30, 40, 50) Gy using Cobalt 60 irradiation (^{60}Co) by irradiator gamma chamber 4000 A at the Center for Application of Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN), Jakarta, Indonesia. The replication for each irradiation dose was 12 times. The observation was conducted to find out the percentage of plant death. The successful mutant plant was detected in 20 Gy for further plantlet mutant generation and analysis.

2.3 Sample Extraction

The leaves and stems from mutant clones and the controls were separated and washed. The leaves and tubers were dried and weighed. All samples were pulverized into powder, and soaked using 96% ethanol. The extraction was done 2 times, each with 100 mL of solvent using signification tool and macerated overnight. The ethanol extract was filtered with Whatman filter paper no. 41 and evaporated by rotary evaporator.

2.4 GC-MS Analysis

The samples extract were dissolved in pure ethanol then injected into the column with conditions 5 µl injection volume with 5 : 1 split ratio, 250°C injection temperature. The helium was used as a carrier gas with a flow/velocity rate of 0.8 µl/min. The column oven temperature was initially set at 70°C and then increased at 5°C/min until reached 200°C, then remained for 1 minute and further increased again at 20°C/min until reached 280°C. The temperature was remained constant for 28 minutes. Mass spectrometer was operated in electron impact ionisation mode with 70 eV voltage. Mass spectral scan range was set at 40-700 (*m*/z). The MS start time was 3 min, and end time was 35 min with solvent cut time was of 3 min.

2.5 Data Analysis

The identification of bioactive compounds was done by comparing the MS fragmentation pattern profile to the NIST database. The relative percentage amount of each compound was calculated by comparing its average peak area to the total areas. The name, molecular weight, and structure of the bioactive compounds of the test materials were confirmed.

3.0 RESULTS AND DISCUSSION

The measurement results of the wet weight and dry weight of rodent tuber mutant leaves and tubers were presented in Table 1. The results of the wet weight and dry weight in the leaves and in the tubers revealed that differences growth response from 23 mutant clones with the control. Table 1 showed that wet and dry weight of 8 mutant clones were bigger than the control, which were clone 20-1-1-1-3, 20-1-2-2-3, 20-1-2-2-1-2, 20-1-2-2-1-3, 20-1-2-4-5, 20-1-2-1-2-2, 20-1-1-2-2-3, and 20-1-3-4-4-8. The growth of 8 clones has a better growth rate compared to the control which it causes the biomass is also greater. The similar results from research by Sriagtala *et al.* [27] showed that gamma ray irradiation treatment, increasing the wet weight and the dry weight of the sorghum plant.

Table 1 Wet weight and dry weight of rodent tuber mutant clones

No.	Mutant clones	Total wet weight (g)	Dry weight (g)		
			Tuber	Leaf	Total
1	Control	104.48	39.62	1.15	40.77
2	20-1-2-4-6	58.5	6.53	3.6	10.13
3	20-1-1-1-3	120.4	42.62	1.26	43.88

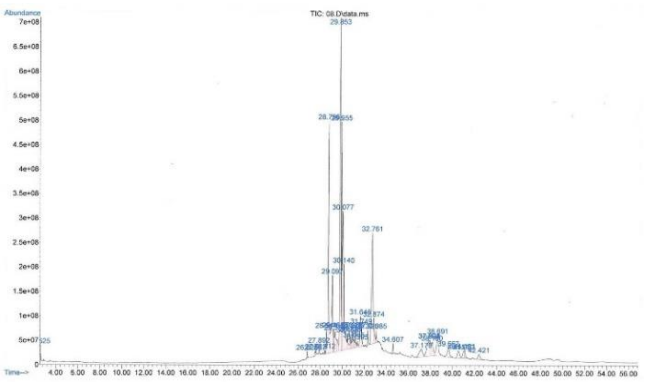
No.	Mutant clones	Total wet weight (g)	Dry weight (g)		
			Tuber	Leaf	Total
4	20-1-2-1-1-3	83	7.08	5.41	12.49
5	20-1-2-1-2-6	35	7.88	0.99	8.87
6	20-4-2-1-1-1	96.4	18.04	3.43	21.47
7	20-1-3-4-4-9	45.6	11.33	0.82	12.15
8	20-1-2-2-3	106.2	87.01	6.66	93.67
9	20-1-2-1-2-6	162.1	34.34	5.61	39.95
10	20-1-2-2-4-3	77.7	26.42	0.94	27.36
11	20-1-2-2-1-2	211.9	53.17	5.58	58.75
12	20-1-2-2-1-3	241.5	56.09	6.69	62.78
13	20-1-2-4-5	184.9	71.51	0.55	72.06
14	20-1-2-4-9	81.7	19.32	2.2	21.52
15	20-1-3-1-4-3	29.5	4.1	1.17	5.27
16	20-1-2-1-2-2	167	47.97	4.95	52.92
17	20-1-1-2-2-3	247	50.97	9.18	60.14
18	20-1-2-1-2-6	30.2	12.7	0.7	13.40
19	20-1-2-2-1-2	23.5	4.01	0.91	4.92
20	20-1-2-1-2	61.1	18.57	1.07	19.64
21	20-1-1-3-2-4	77.8	22.05	0.96	23.01
22	20-1-3-4-4-8	178.3	71.78	1.4	73.18
23	20-1-2-4-4	88.4	36	0.34	36.34
24	20-1-1-2-1	95.1	26.88	1.95	28.84

The effect of gamma ray irradiation treatment also seen from the morphology of mutant plant with control plant, such as the leaf color is greener as well as tuber shape (Figure 1 and 2). Several mutant clones have a leaf color that was greener and have wider leaf than the control. Jan *et al.* [14] stated that gamma irradiation at 10 kGY can increase the total amount of b chlorophyll in *Cullen corylifolium* (L.) to 71.66 % compared to the control.

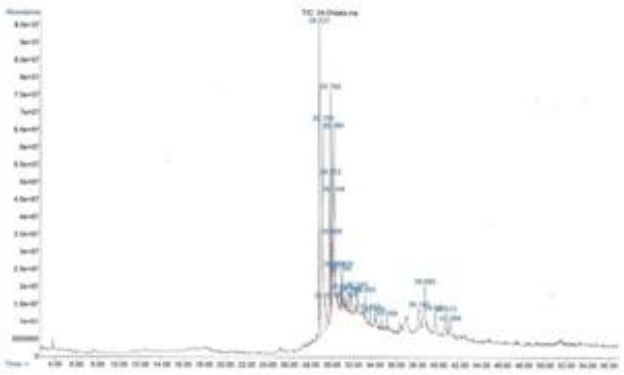
The significant difference between mutant and control plant was seen from the tuber shape. Rodent tuber that has not been treated with gamma irradiation (control) has smaller tuber and round-shape, while the tuber of mutant plant mostly has bigger tuber and oval-shape (Figure 2). The research of Yalindua *et al.* [33] on cultivars yam (*Dioscorea alata* L.) showed that gamma ray irradiation could induce variation in tuber shape from the previous round shape become elongated. Rosmala *et al.* [25] showed that gamma irradiation treatment in the handaleum plants can affect the anatomy of leaves and the diameter of stems.

(a) Control plant (b) Mutan clone (PM 1 = 20-1-1-2-2-3)

Figure 1 The morphological appearances between control and mutant clone



(a) GC-MS chromatogram of control plant



(b) GC-MS chromatogram of mutant clone (PM 5 = 20-1-1-1-3)

Figure 2 The differences of GC-MS analysis chromatogram from control and mutant clone in the tuber

GC-MS analysis showed the differences of bioactive compound in the leaves and the tubers which leaves contained 10 bioactive compounds and tubers only contained 7 bioactive compounds. Generally, the bioactive compounds in mutant leaves were greater than the control. The highest octadecadienoic acid conducted from clones 20-1-2-2-1-3. The 20-1-2-4-5 clone also contains campestrol and beta stigmasterol 3 times than the control. Among 8 mutant clones that were analyzed, there is differences number of bioactive compounds in leaves and tubers. Several bioactive compounds in the mutant plant had increased rather than the control. Mutant plant also contains several types of anticancer compounds that cannot be found in control plant (Table 2 and 3).

Gamma ray irradiation would produce reactive free radical and when interacted with DNA, it can change the structure of the nucleotide base and release the double bonds. This process can cause the mutation, such as deletion, invertion, and duplication of damage sites. The mutation is a way to repair the double bonds of DNA damage [10]. In the end, the DNA mutation changes the protein expression pattern that will subsequently affect the production of chemical content (secondary metabolites).

GC-MS method was used to analyze the compound from the leaves and tubers of the rodent tuber (control and mutant). The mutant clones were analyzed for 20-1-1-2-2-3, 20-1-2-2-1-3, 20-1-2-1-2-2,

20-1-2-4-5, 20-4-2-1-1-1, 20-1-2-1-2-6, 20-1-2-2-4-3, and 20-1-2-2-4-3 (Table 2 and 3). Rodent tuber mutant's leaves contain 8 anticancer compounds that the relative amount is higher than the relative amount of the compound in the control plants. The mutant plant's leaves also contain 2 new anticancer compounds that did not find in the control, which were phytol isomer and eicosane (Table 2).

The various bioactive compounds were confirmed in the rodent tuber mutant clones which have antimicrobial and antioxidants function such as hexadecanoic, eicosane, and squalene [16], phytol [22, 26], pyridine carboxamide [29], octacosane [4], octadecanoic acid [7].

Tubers of rodent tuber mutant contain 5 new anticancer compounds that were not found in the control, which were hexadecanoic acid ethyl ester, octadecadienoic acid, squalene, beta sitosterol, eicosane, and octacosane. Moreover stigmasterol content from the mutant tuber was higher than the control (Table 3).

Hexadecanoic acid, methyl ester has been reported to possess anti-inflammatory, antioxidant, hypocholesterolemic, 5-alpha reductase inhibitor, nematicide, pesticide and antiandrogenic [34], and also had cytotoxic activity against leukemia, cancer cell MOLT-4 and antitumor [11].

Table 2 The comparison of relative percentage of bioactive compounds in the leaves of rodent tuber mutant and control based on GC-MS analysis

No.	Compound name	Relative percentage (%)							
		K	PM 1	PM 2	PM 3	PM 4	PM 5	PM 6	PM 7
1	Hexadecanoic acid ethyl ester	0.14	NI	0.35	NI	NI	0.21	0.35	0.17
2	Hexadecanoic acid	25.52	21.05	27.7	14.78	18.06	21.09	23.69	23.54
3	Octadecadienoic acid v	5.52	NI	36.75	18.39	12.42	NI	NI	0.99
4	Squalene	2.24	2.63	1.79	4.73	4.87	1.65	1.83	NI
5	Campesterol (ergost-5-en-3-ol)	2.07	NI	2.22	NI	6.33	0.7	2.81	1.49
6	Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol)	4.21	5.27	2.32	9.43	9.45	1.89	5.43	2.96
7	Stigmast-5-en-3-ol (beta-sitosterol)	0.94	NI	0.86	1.29	NI	NI	NI	1.04
8	Phytol isomer	NI	5.84	6.75	NI	7.14	NI	NI	NI
9	Pyridine-3-carboxamide,oxime	0.23	NI	NI	2.74	NI	NI	NI	NI
10	Eicosane (Icosane)	NI	NI	NI	NI	NI	1.19	NI	NI

Note: NI is not identified. All confirmed through NIST database. K = Control, PM 1 = 20-1-1-2-2-3, PM 2 = 20-1-2-2-1-3, PM 3 = 20-1-2-1-2-2, PM 4 = 20-1-2-4-5, PM 5 = 20-1-1-1-3, PM 6 = 20-4-2-1-1-1, PM 7 = 20-1-2-1-2-6.

Table 3 The Comparison of relative percentage of bioactive compound in the tuber of rodent tuber control and mutant based on GC-MS analysis

No.	Compound name	Relative percentage (%)									
		K	PM 1	PM 2	PM 3	PM 4	PM 5	PM 6	PM 7	PM 8	PM 9
1	Hexadecanoic acid ethyl ester	NI	3.34	4.58	3.79	5.34	10.22	8.09	2.8	4.13	7.02
2	Octadecadienoic acid	NI	43	39.25	35.86	21.8	21.11	22.1	5.21	131.5	25.91
3	Squalene	NI	0.97	0.89	1.76	1.28	0.66	0.88	NI	2.58	1.55
4	Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol)	2.49	2.99	3.68	2.82	13.4	5.67	8.56	NI	4.76	4.16
5	Stigmast-5-en-3-ol (beta-sitosterol)	1.3	0.52	0.61	NI	NI	3.53	1.55	NI	NI	NI
6	Eicosane (Icosane)	NI	NI	NI	NI	NI	NI	NI	NI	NI	0.99
7	Octacosane	NI	NI	NI	NI	NI	NI	NI	NI	1.07	NI

Note: NI is not identified. All confirmed through NIST database. K = Control, PM 1 = 20-1-1-2-2-3, PM 2 = 20-1-2-2-1-3, PM 3 = 20-1-2-1-2-2, PM 4 = 20-1-2-4-5, PM 5 = 20-1-1-1-3, PM 6 = 20-4-2-1-1-1, PM 7 = 20-1-2-1-2-6, PM 8 = 20-1-2-2-4-3, PM 9 = 20-1-2-2-3.

Stigmast-5-en-3-ol (3.beta., 24s) (beta-sitosterol) is a phytosterol with functioning as an anti-diabetic [28], and inhibiting cancer cells [5, 32]. Ergost-5-en-3-ol (3 beta) (campesterol) is a phytosterol that able to prevent various cancer cells, including lung cancer [18], gastric cancer [3], and ovary cancer [17].

Octadecanoic acid, methyl ester is known to possess antimicrobial and anti-fungal properties [7], besides that octadecenoic acid ethyl ester has been reported to inhibit the proliferative effect in keloid fibroblasts [21]. Eicosane described as arachidic acid is known for its cytotoxic effects, especially as antimicrobial and antitumor agents [13].

Squalene has been proven to be able to inhibit carcinogenesis of various cancer cells, including colon cancer [24]. Stigmasterol could reduce the number of tumor cells Ehrich Ascites Carcinome (EAC). Stigmasterol is an antioxidant because it could reduce lipid peroxidation and increase gluthathionin, superoxide dismutase, and catalase in EAC mice liver [6]. Young-Sang *et al*. was investigated stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol) had induced to apoptosis in human hepatoma HePG2 cells after 24 h of treatment [34].

4.0 CONCLUSION

This study revealed that gamma irradiation can increase numbers of bioactive compound in the mutant plants. 5 new anticancer compounds were found in the tuber of rodent tuber mutant clones which did not find in the control, for example hexadecanoic acid ethyl ester, octadecadienoic acid, squalene, beta sitosterol, eicosane, and octacosane. Phytol isomer and eicosane were found as new anticancer compounds in the leaves of the rodent tuber of mutant clones. Stigmasterol was found 2-4 times higher than the control.

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