Determination of Bioactive Compounds of Superior Mutant Rodent Tuber (*Typhoniumflagelliforme*) in Various Fractions Using GC-MS

Nesti Fronika Sianipar¹,²*, Khoirunnisa Assidqi¹,², Yuni Elsa Hadisaputri³, Supriatno Salam⁴, Romesta Tarigan², Ragapadmi Purmananingsih⁵

¹Food Technology Department, Faculty of Engineering, Bina Nusantara University, Jakarta 11480, Indonesia
²Research Interest Group Food Biotechnology, Bina Nusantara University, Jakarta 11480, Indonesia
³Department of Pharmaceutical Biology, Faculty of Pharmacy, Padjajaran University, Jatinangor 45363, Indonesia
⁴Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjajaran University, Jatinangor 45363, Indonesia
⁵Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biögen), Bogor 16111, Indonesia

*Correspondence author: nsianipar@binus.edu

Abstract. Superior mutant rodent tuber plant (*Typhoniumflagelliforme*) is a medicinal herb of Indonesia, which immensely useful for anticancer activity. Some studies reported that the leaves and the tubers, conventionally parts of rodent tuber plant, showed various anticancer, antimicrobial, and antioxidant potential. This study aim is to determine the bioactive compounds of superior mutant rodent tuber plants through n-hexane and ethyl acetate fractions using GC-MS for the analysis. Phytochemical characterization of the superior mutant rodent tuber plant extracts was detected by qualitative analysis. A total of 20 bioactive compounds were obtained in an n-hexane fraction. A total of 4 bioactive compounds were identified in ethyl acetate fraction. The GC-MS analysis showed the presence of the major compound in ethyl acetate and n-hexane fraction were 9,11-octadecadienoic acid, methyl ester (55.37%), 9-octadecenoic acid (Z)-methyl ester (26.77%), hexadecanoic acid, methyl ester (2.97%), 9,12-octadecadienoyl chloride (2.47%), humulene (1.11%), octahydronapthalen (0.97%), alloaromadendrene oxide (2) (0.85%), pentadecanoic acid, methyl ester (0.80%), methyl tetradecanoate (0.79%), and eucalyptol (0.69%). Most of the identified compounds in the n-hexane and ethyl acetate fractions exhibit following biology activity, such as anticancer, antioxidant, anti-androgenic, antimicrobial, antifungal, and anti-inflammatory. This study provides an overview of the chemical compounds and their beneficial impact on developing drugs from n-hexane and ethyl acetate fraction of the superior mutant rodent tuber plant.

1. Introduction

Rodent tuber (*Typhoniumflagelliforme*) is a herbal plant originating from Indonesia. This plant is spread in various countries such as Sri Lanka, India, Australia, and multiple countries in Asia. This plant has been used traditionally as an anticancer drug. The parts of rodent tuber plants often used as...
medicines are tubers and plant leaves [1]. Extracts from rodent tuber have been identified to have anticancer properties in colon cancer [2], breast cancer [3], lung cancer [4], and leukemia [5]. The main compound groups in rodent tuber plants are flavonoids, steroids, tannins, saponins, and glycosides [6,7].

The differences in the quantity of bioactive compounds in a plant can be increased by increasing genetic diversity, such as mutations, introduction, exploration, hybridization (crossing), and gene transfer. The mutation technique with gamma-ray irradiation at a 6 graysdose has been carried out on a population of rodent tuber callus somatic cells. The mutation results showed the diversity of rodent tuber's putative shoot growth in vitro [8]. The plant diversity was identified using Randomly Amplified Polymorphic DNA (RAPD) molecular markers [9]. The sixth-generation putative mutant (MV6) in the ethanol extract of rodent tuber plants also showed differences in chemical compounds' composition with the mother rodent tuber plant extract [10].

In general, based on the level of polarity, ethanol is a polar solvent that can only attract polar chemical compounds. Meanwhile, to bind non-polar compounds in rodent tuber extract are needed non-polar solvents such as n-hexane and n-butanol. Chemical compounds that have semi-polar (polar and non-polar) properties are dissolved with ethyl acetate. The process of separating compounds based on polarity properties can be through fractionation techniques [11]. Analysis of chemical compounds in non-polar and semi-polar solvent fractionation needs to be carried out on superior mutant rodent tuber plants to determine new compounds and chemical composition differences. Various chemical compounds found in mutant rodent tuber are a class of fatty acid compounds that can be antiproliferative and cause apoptosis in cancer cells [12].

The main techniques for identifying chemical compounds are separation and molecular detection. The separation technique that is usually used is based on differences in molecular motion patterns on gas media (Gas Chromatography/GC). Furthermore, the separated compounds are detected using Mass Spectrometry (MS). This method analyzes compounds based on their atomic and molecular weight. The combination of gas chromatography techniques and mass spectrometry is called GC-MS [13]. The advantages of the GC-MS instrument are efficiency, fast analysis, small sample size, high resolution, and increased sensitivity [14]. This method has been widely used in analyzing active compounds in various medicinal plants, such as stone leaf (Tetracera scandens) [15], ginger (Zingiber officinale) [16], and curcuma (Curcuma xanthorrhiza) [17].

The utilization of GC-MS in determining chemical compounds in superior mutant rodent tuber will characterize the bioactive compounds that have a role in biological activity. The GC-MS instrument can detect primary metabolites and volatile metabolites [18]. Primary metabolites consist of organic acids, amino acids, and sugars, while volatile metabolites such as terpenoids, benzoates, and aromatic compounds [19]. Identifying this chemical profile will be very useful in the development of anticancer drugs from the diversity of rodent tuber, especially in the superior mutant rodent tuber. Rodent tuber, which has a higher quantity and quality of new compounds, can be developed as an anticancer drug. This study analyzes the content of bioactive compounds in superior mutant rodent tubers that have been fractionated with n-hexane and ethyl acetate using the GC-MS instrument.

2. Materials and Methods

2.1. The extraction and fractionation of superior mutant rodent tuber plant

Sianipar&Purnamaningsih's collection has a superior rodent tuber mutant plant, which grows at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biogen), Bogor, Indonesia. The tubers of superior rodent tuber mutant were first harvested, washed, and dried at 40°C using the oven. The powder of the tubers was macerated in 96% ethanol at six times until not concentrate. The filtrate was filtered through Whatman filter paper No. 1. The filtrate was evaporated to dryness using Buchi Rotavapor Dynamic R-300 at 50°C. The concentrated extract was collected and used for the next step of fractionation. The crude extract of ethanol superior
rodent tuber mutant was treated for the fractionation process with n-hexane, ethyl acetate, n-butanol, and water. The various fractions were obtained using a separating funnel.

2.2. Phytochemical screening of superior mutant rodent tuber plant fractions
All the various fractions were conducted phytochemical screening to detect the presence of secondary metabolites in the extract. The qualitative analyses were described to detect phenolic, flavonoids, steroids, triterpenes, alkaloids, tannins, and saponins.

2.2.1. Phenolic test
2 gr of the n-hexane and ethyl acetate fractions was extracted with methanol in 5 mL then placed into a test tube. The sample was added and shaken a few drops of 1% gelatin solution containing sodium chloride. Appearance of the bluish-black color indicates the presence of phenolics.

2.2.2. Tannin test
The n-hexane and ethyl acetate fractions was taken 2 gr of weight then extracted with methanol in 5 mL. The sample was added and shaken a few drops of 5% ferric chloride (FeCl₃) solution in the test tube. Formation greenish-black color indicates the presence of tannins.

2.2.3. Triterpen and steroid test
The sample of fractions were extracted with ethanol at 5 mL. The extract was dried and extracted again with the mixture ratio of chloroform and water (1:1). The chloroform extract in the test tube was added by a few drops of concentrated sulfuric acid and anhydrite acetic acid. The color will be changing into red or brown indicates the presence of triterpene. The blue, purple or green color shows the existence of steroids.

2.2.4. Flavonoid test
All fraction samples were extracted with methanol in 5 mL. A few drops of HCl (hydrochloric acid) then added Mg powder and shake vigorously to see if foam forms. The sample will contain flavonoids if there is foam with a lot of intensity, and the solution turns orange or pink color.

2.2.5. Alkaloid test
2 gr of fraction samples with 5 mL of ammonia chloroform was transferred into four differences of test tube. The first test tube was added 1 ml of Dragendorf's reagent (potassium bismuth iodide solution). An orange-red precipitate is present in the alkaloids. The second test tube was dropped 1 mL of Wagner's reagent (potassium iodide). The significant alkaloids indicated by reddish-brown precipitate. 1 mL of Mayer's reagent (potassium mercuric iodide solution) was added into the third test tube. The whitish or cream precipitate indicates the presence of alkaloids. The Hager's reagent (saturated ferric solution) was added into the fourth test tube about 1 mL. The yellow-colored indicates it contains alkaloids.

2.2.6. Saponin test
The extracted sample with 5 mL of ammonia chloroform was transferred into a test tube then shake vigorously and let it stand for 2 min. Afterwards, 2 drops of HCl was added and shaken vigorously and see if any foam forms after 10 min. It contains saponins if there is foam with high intensity and consistent for 10 min.

2.3. GC-MS analysis of superior mutant rodent tuber plant fractions
The sample of n-hexane and ethyl acetate fractions were analyzed for the chemical compounds using GC-MS. Gas Chromatograph column interfaced into a Mass Spectrometer. Agilent Technologies 5977B was equipped with a split injection for the sample used introduction. The initial oven temperature was raised at 70°C until reached at 260°C with 6°C/min. The carrier gas was used as a
helium gas with the flow rate at 1.0 mL/min. The peaks were obtained to analyze the mass spectral. The relative molecular mass and molecular weight was matched and compared against NIST and PubChem NCBI library to identify chemical compounds.

3. Results and Discussion

Determination of the group of chemical compounds based on the level of polarity has been carried out in several superior mutant rodent tuber plant fractionations of n-hexane, ethyl acetate, n-butanol, and water (Table 1). The n-hexane and ethyl acetate fractions revealed several groups of secondary metabolites. Qualitatively, the chemical compounds produced, as shown in Table 1, are phenolics, flavonoids, steroids, triterpenes, alkaloids, tannins, and saponins. The main group of chemical compounds found in the fractionation of ethyl acetate is flavonoids. According to Ahmed et al. [53], flavonoids have an active role as anticancer and neuroprotective agents in Hep2 and MCF-7 cancer cells. Previous studies have also suggested that flavonoids are responsible for various pharmacological bases due to their ability to be associated with antioxidants and anti-inflammatory properties [20]. The fractionation results on n-hexane were screened and found two main chemical compounds, such as steroids and triterpenes. Triterpenes and steroids have been found to have the potential for therapeutic agents to fight tumorsonestrogen receptors and prostate cancer [21, 22].

Table 1. The qualitative of chemical compound types in various fractionation of superior mutant rodent tuber plant

| Compound group | Water | n-Butanol | Ethyl Acetate | n-Hexane |
|----------------|-------|-----------|---------------|----------|
| Phenolic       | -     | -         | +             | +        |
| Flavonoids     | -     | +         | ++            | -        |
| Steroids       | --    | -         | +             | ++       |
| Triterpenes    | --    | -         | +             | ++       |
| Alkaloids      | -     | +         | +             | +        |
| Tannins        | ++    | +         | -             | -        |
| Saponins       | ++    | ++        | -             | -        |

Note: + = presence; − = absent.

The chromatogram results on the n-hexane fraction of the superior mutant rodent tuber plant using GC-MS analysis are shown in Figure 1. The quantification of volatile chemical compounds in the n-hexane fraction is shown in Table 2. Total bioactive compounds found in n-hexane fractionation as much as 20 bioactive compounds. 9,11-octadecadienoic acid, methyl ester is the major compound that has been found with 55.37% followed by 9-octadecenoic acid (Z) - methyl ester (26.77%), humulene (1.11%), octahydropentalene (0.97%), 2,4-Di-tert-butylphenol (0.91%), pentadecanoic acid, methyl ester (0.80%), 9,12-octadecadienoyl chloride, (Z, Z) (0.77%), methyl tetradecanoate (0.79%), azulene (0.69%), gamma-muurolene (0.64%), eucalyptol (0.69%), isoborneol (0.54%). Chromatogram and spectra analysis of ethyl acetate fractionation resulted in 4 bioactive compounds, shown in Figure 2 and Table 3. Major compounds found in the ethyl acetate fraction are hexadecanoic acid, methyl ester; 9,12-octadecadienoyl chloride, (Z, Z); and 2,3-butanediol with percentages of 2.97%, 2.47%, and 1.51%, respectively.
Figure 1. Determination of chromatogram of n-hexane fraction of superior mutant rodent tuber using GC-MS

Table 2. The bioactive compounds of the chromatographic n-hexane fraction of superior mutant rodent tuber plant using GC-MS analysis

| No. | RT   | m/z       | Compound name                                                                 | Area% | Quality |
|-----|------|-----------|-------------------------------------------------------------------------------|-------|---------|
| 1   | 12.464 | 154.1358 | Eucalyptol                                                                   | 0.69  | 43.8    |
| 2   | 14.860 | 150.1201 | Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl                                   | 1.60  | 53.8    |
| 3   | 15.237 | 154.1359 | Isoborneol                                                                   | 0.54  | 28.9    |
| 4   | 15.784 | 154.1355 | Cyclohexane, 1-metanol-4-trimethyl                                           | 0.48  | 43.1    |
| 5   | 18.786 | 204.1878 | Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylthienyl)-, [1S-(1.alpha.2.beta.,4.beta.)] | 2.30  | 76.8    |
| 6   | 19.066 | 190.1722 | Caryophyllene                                                                | 0.71  | 77.4    |
| 7   | 19.330 | 204.1879 | (E)-beta-Famesene                                                            | 2.13  | 69.0    |
| 8   | 19.432 | 204.1877 | Azulene, 1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylthienyl)-,[1R-(1.alpha.,3a.beta.,4.al] | 0.69  | 76.9    |
| 9   | 19.556 | 204.1878 | Humulene                                                                     | 1.11  | 87.1    |
| 10  | 19.726 | 204.1878 | 4a,8-Dimethyl-2-(prop-1-3n-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene (R,Z)-2-Methyl-6-(4-methylcyclohexa-1,4-dien-1-yl)hept-2-en-1-ol | 0.97  | 88.0    |
| 11  | 19.801 | 220.1827 | Methyl tetradecanoate                                                         | 1.16  | 59.2    |
| 12  | 19.848 | 204.1878 | 9,12-Octadecadienoyl chloride, (Z,Z)-methyl ester (E,E)                     | 0.71  | 65.2    |
| 13  | 19.877 | 204.1878 | 9,11-Octadecadienoic acid, methyl ester (E,E)                                | 0.14  | 65.5    |
| 14  | 20.235 | 206.1671 | 2,4-Di-tetra-3-butylphenol                                                   | 0.91  | 87.2    |
| 15  | 22.617 | 242.2246 | Methyl tetradecanoate                                                         | 0.79  | 87.1    |
| 16  | 22.917 | 220.1827 | Alloaromadendrene oxide-(2)                                                  | 0.85  | 71.2    |
| 17  | 23.721 | 256.2402 | Pentadecanoic acid, methyl ester                                             | 0.80  | 51.0    |
| 18  | 25.627 | 298.2063 | 9,12-Octadecadienoyl chloride, (Z,Z)                                        | 0.77  | 91.0    |
| 19  | 26.464 | 270.2559 | 9,11-Octadecadienoic acid, methyl ester (E,E)                                | 0.55  | 81.3    |
| 20  | 26.560 | 310.2872 | 9-Octadecenoic acid (Z)-methyl ester                                         | 26.77 | 91.1    |

Note: quality is compared with pure compound.
Figure 2. Determination of chromatogram of ethyl acetate fraction of superior mutant rodent tuber using GC-MS

Table 3. The bioactive compounds of the chromatographic ethyl acetate fraction of superior mutant rodent tuber plant using GC-MS analysis

| No. | RT   | m/z     | Compound name                                           | Area% | Quality |
|-----|------|---------|---------------------------------------------------------|-------|---------|
| 1   | 4.568| 90,0681 | 2,3-Butanediol,[S-(R*R*)]                                | 1.51  | 40.7    |
| 2   | 24.788| 270,2559| Hexadecanoic acid, methyl ester                         | 2.97  | 77.3    |
| 3   | 26.444| 298,2063| 9,12-Octadecadienoyl chloride, (Z,Z)                    | 2.47  | 90.9    |
| 4   | 32.043| 390,2770| Bis(2-ethylhexyl) phthalate                              | 93.06 | 85.7    |

Note: quality is compared with pure compound.

Several bioactive compounds produced from the fractionation of n-hexane and ethyl acetate have several biological activities, including antioxidant, anticancer, anti-inflammatory properties, as shown in Table 4. Summary results of the chemical compounds' overall structure from the fractionation of n-hexane and ethyl acetate are presented in Figure 3. This study indicates that more bioactive compounds were found in the n-hexane fractionation of the superior mutant rodent tuber plant using the GC-MS analysis method.

In general, the GC-MS results showed that the n-hexane fractionation of the superior mutant rodent tuber contains many groups of volatile compounds such as terpenoids and fatty acids, which have biological activity. One of the volatile bioactive compounds, including monoterpenes and sesquiterpenes, is eucalyptol and gamma-muurolene [54]. There are many groups of volatile compounds in the ethyl acetate fraction, which found fatty acid compounds. Bioactive compounds included in the fatty acid compounds are hexadecanoic acid, methyl ester; 9-Octadecenoic acid (Z) - , methyl ester; pentadecanoic acid, methyl ester; 9,12-Octadecadienoyl chloride, (Z, Z); and methyl tetradecanoate.

The terpenoid group of compounds, such as eucalyptol, has a toxicity effect against H1299 cells at the percentage level of cell viability with IC<sub>10</sub>. This involves enzyme activity in antioxidant mechanisms that are useful for anticancer protection [23]. According to a study reported by Bhattacharjee & Chatterjee [24], proapoptotic, anti-inflammatory, and anti-tumor activities were identified, which were confirmed by in vivo and in vitro studies. Gamma-muurolene compound is a chemical composition of essential oil compounds from the sesquiterpene compound group. Sesquiterpenes have a wide range of biological activities, including anti-inflammatory, antimicrobial, antioxidant, and cholinesterase activity inhibitor activity [25, 26].
This study shows that palmitic acid, elaidic acid, linoleic acid, and myristic acid, which are included in the fatty acid compound group, can inhibit the DNA synthesis of breast cancer cells and inhibit metastatic growth and tumor cell necrosis [27, 28]. Palmitic acid is one of the most common saturated fatty acids found in plants, which has been shown to induce apoptosis in AR42J pancreatic exocrine cells. Myristic acid, stearic acid, and arachidic acid significantly caused AR42J cell death at concentrations higher than 10 µM [29]. This study has proven that the fractionation of n-hexane can be used as a bioactive compound that positively correlates to biological activity, especially in clinical trials in the development of anticancer drugs.

Table 4. The resume of bioactive compounds as biological activity of superior mutant rodent tuber plant of ethyl acetate and n-hexane fractions

| Biological activities                        | Compound Name                        | Compound Nature       | Reference |
|---------------------------------------------|--------------------------------------|-----------------------|-----------|
| Anticancer, antimicrobial, anti-inflammatory | Hexadecanoic acid, methyl ester      | Fatty acid            | [30-32]   |
| Anticancer, anti-inflammatory               | 9-Octadecenoic acid (Z),methyl ester | Omega-9/Elaidic acid  | [33, 34]  |
| Antitumor                                   | 2,3-Butanediol,[S-(R*R*)]            | Alcohol group         | [35]      |
| Anticancer, antioxidant                     | Eucalyptol                           | Essential oil         | [36, 37]  |
| Antioxidant, antitumor                      | Isoborneol                           | Phenol                | [38, 39]  |
| Anti-inflammatory, anticancer, antifungal   | 2,4-Di-tert-butylphenol              | Phenol                | [40, 41]  |
| Antioxidant, anticancer, allelophatic activity| Alloaromadendrene oxide-(2)         | -                     | [42, 51]  |
| Antioxidant                                 | Pentadecanoic acid, methyl ester     | Fatty acid            | [45]      |
| Anticancer                                  | Methyl tetradecanoate                | Myristic acid         | [46]      |
| Antiproliferative, anticancer               | Humulene                             | -                     | [43, 44]  |
| Antioxidant                                 | Azulene, 1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylthene)-,[1R-(1.alpha.,3a.beta.,4.al| -                     | [47]      |
| Anti-androgenic                             | 9,12-Octadecadienoyl chloride, (Z,Z)| Linoleic acid         | [48]      |
| Antioxidant, anti-inflammatory              | 4a,8-Dimethyl-2-(prop-1-3n-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene| Essential oil         | [49, 50]  |
| Antitumor                                   | -                                    | -                     | [52]      |
Figure 3. The structure chemical compounds of of superior mutant rodent tuber plant of ethyl acetate and \textit{n}-hexane fractions which has biological activities

4. Conclusion

Based on the phytochemical screening, the compound groups were present alkaloids, phenolic, triterpenes, steroids in the \textit{n}-hexane and ethyl acetate fractions. The \textit{n}-butanol fraction had a major group of chemical compounds was saponins. The major group of chemical compounds from the ethyl acetate fraction was flavonoid. GC-MS analysis was determined bioactive compounds from the \textit{n}-hexane and ethyl acetate fractions of superior mutant rodent tuber plant. The bioactive compounds from \textit{n}-hexane fraction which has big potential for anticancer and antioxidant were identified as 9,12-octadecadienoyl chloride; 9,11-octadecadienoic acid, methyl ester; methyl tetradecanoate; octahydropaphthalene; 9-octadecenoic acid (Z), methyl ester; eucalyptol; humulene; alloaromadendrene oxide-(2), gamma-muurolene, isoborneol, pentadecanoic acid, methyl ester; and azulene. The bioactive compounds in ethyl acetate fraction were found hexadecanoic acid, methyl ester potenst as anticancer, antimicrobial, and anti-inflammatory. Further studies are needed to
explore the potential of the bioactive compounds from the n-hexane and ethyl acetate fractions of superior mutant rodent tuber plantin biological activity and toxicity profiles.

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