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Research Article

THE YIELD OF CHEMICAL COMPOUNDS OF BLACK SOYBEANS (*Glycine soja* (L.)) MUTANT INDUCED BY EMS AND GAMMA RAYS INDUCTION

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ABSTRACT. Black soybeans have a higher flavonoid content than yellow soybeans. Flavonoids are phenolic compounds which can potentially be used as antioxidants. The quality of black soybean needs to be increased by plant breeding. As previous research has demonstrated, mutation treatment can increase the levels of flavonoids, antioxidant activity, and phenolic compounds. This research aims to to determine the levels of flavonoids, antioxidant activity and phenol of black soybean (Detam3 varieties) by gamma rays and Ethyl Methanesulphonate (EMS) induction. It employed Completely Randomized Design (CRD). Doses used in this research were 1000 Gy gamma rays, 1% EMS, and combinations. Total phenolics were determined using folin-ciocalteu method, expressed as gallic acid equivalent (GAE)/gr of extract. Total flavonoids were determined using $AlCl_3$ method, expressed as quercetin equivalent (QE)/gr of extract. Meanwhile, antioxidant activities were determined using Ferric Reducing Antioxidant Power (FRAP), expressed as ascorbic acid equivalent (AAE)/gr of extract. the highest flavonoid level at 185.748 QE/g extract, antioxidant activity at 166.752 mg AAE/g extract, and total of phenolic compound at 645.447 mg GAE/g extract

Keywords: *black soybean, gamma rasy, ethyl methanesulfonate, chemical compound, mutant*

INTRODUCTION

Black Soybeans play an important role in food industry, especially soy sauce industry. In terms of its nutritional contents, black soybeans are superior to yellow soybeans. According to [1]. The availability of black soybeans is decreasing because farmers prefer to plant yellow soybeans which have bigger seed and higher productivity. Based on BPS data, Indonesian average production of Black soybean in 2016 was around 950.000 kg/year, while the demand for the soybeans is about 2.6 million tons/year. The domestic production can only meet 40% of the needs, while the other 60% were imported. Detam 3 is one of Black Soybean varieties that has higher levels of protein and antioxidants compared to other varieties.

Antioxidant contents which can be found in plants are polyphenol, carotenoid, and vitamin [2]. Flavonoid is one of secondary metabolisms from polyphenol group produced by plants [3]. This compound is a scavenger of free radicals and has ability to inhibit lipid oxidations [4]. The antioxidant activity of phenol and flavonoids which can be activated by reducing free radicals depends on the number of hydroxyl groups of their molecular structure [5]. Phenol and flavonoid compouds play many roles in

biological activity such as anti-oxidant, anti-inflamoatory, anti-aging, anti-bacterial, and anti-tumor.

In order to increase genetic variations, including the quantity and quality of Black Soybean production, both the goverment and researchers need to implement plant breeding program. A population with a high genetic variation is needed to make new varieties. It can be obtained by introduction, breeding, mutation, and genetic transformation. Black Soybeans have very small flowers. Thus, hybridization is difficult and expensive so that mutagenesis is induced to increase the genetic variability rapidly [6]. Assembling superior soybean varieties can be done by physical mutation technology such as gamma ray irradiation and by using chemical compounds like EMS. Mutation techniques can broaden the genetic diversity of plants [7], increasing the chances of successful selection and selection line which should be in line with the purpose of plant breeding.

Research on the effect of gamma ray to Soybean by Papovic et al. [8] had shown that 1000 Gy gamma ray irradiation increased the antioxidant activity (42.74%), genistein (21.47 mg/kg), daidzein (34.51 mg/kg) and total phenol (2.423 mg kat./g). EMS causes mutations on DNA level by changing the DNA base (causing point mutation), and a slight damage to thr chromosome so that it is beneficial for the plant breeding. Point mutation is also passed down to the next generation. Greene et al. [9] has shown that Arabidopsis which were given EMS 40 mM for 10-20 hours showed 99% mutation in the DNA and found that GC were changed to AT and vice versa. The intensity of mutation is relatively high at 1/3.000 kilos of base or 10 mutation/genom. That 0.5% EMS treatment on soybean is effective for increasing the protein content to 41.15% [10].

The combination of gamma ray irradiation mutagen and EMS is still rarely performed on soybean plants. Research conducted by Satpute [11] on soybean MAUS-71 and JS-335 shows that both mutagens increase the frequency of chlorophyll mutations and sterile pollen. EMS is more effective while gamma ray is more efficient. Soybean Cv. PK 1029 which was given 0.1% EMS treatment had the most mutations and 200 Gy gamma ray treatment was the most efficient in mutation induction [12]. Based on this explanation, it is necessary to observe the total levels of flavonoids, phenols and antioxidant activity of black soybeans induced by gamma ray and EMS irradiation.

MATERIALS AND METHODS

Mutation Induction Using EMS

EMS was dissolved with sodium phosphate buffer 7 and DMSO 4%. The concentration to be used was 1%. The seeds were soaked in phosphate buffer for 6 hours, then soaked in 1% EMS solution for 6 hours, and rinsed with distilled water [13]. As a control (0% EMS), the seeds were soaked in phosphate buffer pH 7 for 6 hours. The treatment was carried out at room temperature.

Mutation Induction Using Gamma Rays

Black Soybeans were put into a transparant plastic then radiated with 1000 Gy of gamma rays using Co-60 Gammacell 220 irradiator at a dose of 4585.5 Gy/hour at Badan Tenaga Nuklir Nasional (BATAN Jakarta).

Detail of Treatment

The detail of treatment: T0 (control-without phosphate buffer immersion), T1 (control-immersion with phosphate buffer), T2 (1000 Gy gamma ray), T3 (EMS 1%), T4 (1000 Gy gamma ray + 1% EMS). Seeds were planted in polybags. In each treatment, 100 seeds were treated until harvest and then analyzed.

Sample Preparation of Ethanolic Extract

One gram of Black soybean seeds was crushed until smooth then filtered. Seed powder was dissolved in ethanol p.a with 1:4 ratio then incubated at room temperature for 24 hours. After that, it was filtered so that the filtrate was separated then evaporated at 45ºC in an oven to obtain an extract.

Preparation of the Solution

Making the quercetin Solution. 10 mg of quercetin (comparator) was dissolved in 100 ml of ethanol p.a as a stock solution. This stock solution was diluted to 6, 8, 10, 12 and 14 ppm as a standard solution.

Making the $AlCl_3$ Solution. 5gr $AlCl_3$ was dissolved in 250 ml of aquades.

Making the sodium scetate solution. Sodium Acetate 2.461 gr dissolved in 250 ml Aquades.

Raw Curve Measurement

Each concentration of Quercetin solution was taken for about 1ml of quercetin solution was taken from each concentration then 1 ml of AlCl3 2% solution and 1 ml of 120 mM sodium acetate were added. Samples were incubated for 60 minutes at room temperature. Measured absorption at 435 nm wavelength was done three times.

Measurement of Flavonoid Total

15 mg of extract was dissolved in 10 ml of ethanol and solution with 1500 ppm concentration was obtained. 1 ml of this solution was taken and then added with 1 ml $AlCl_3$ 2% and 1 ml of 120 mM Sodium Acetate. The sample was incubated for 60 minute at room themperature. The absorbance was measured using UV-vis spechtrophotometry method at 435 nm wavelenght. Three samples were made and acquired the average value of absorbance [14].

Preparation of Solution

Preparation of Na_2CO_3 solution. 7.5 gr Na_2CO_3 was dissolved in 100 mL of aquades.

Preparation of Gallic acid solution. Stock Solution 1000 ppm made from 10 mg Gallic acid was dissolved in 10 mL of aquades. From this stock solution 1, 1.25, 1.5, 1.75, and 2 mL were diluted and homogenized in 10 mL aquades. The concentrations of Gallic acid standard solution were 100, 125, 150, 175 and 200 ppm.

Determination of Total Phenols

Samples with a concentration of 1000 ppm taken 1 mL was taken from samples with a concentration of 0.5 mL Folin-Ciocalteu reagent and 2 mL of sodium carbonate

solution (Na2CO3) 7.5% then homogenized. Solution was incubated for 3 minutes then aquades was added up to 5 mL. The solution was incubated for 60 minutes and the absorption was measured at 778 nm wavelenght. Gallic acid solution was used for the standard solution, while for blanko was replaced with ethanol.

Preparation of Ascorbic Acid Standard Solution

1000 ppm of stock solution was made from 25 mg of ascorbic acid in 1% of oxalic acid up to 25 mL. From this stock solution 0.2, 0.4, 0.6, 0.8, and 1 mL were diluted in 10% of oxalic acid up to 10 mL and homogenized. The concentrations of standard solution were 20, 40, 60, 80, and 100 ppm.

Preparation of Solution

Oxalic 1% solution . This solution was made from 1 gr of oxalic acid dissolved in 100 mL of aquadest

Pottasium ferrisianida 1% solution. This solution was made from 1 gr pottasium ferrisianida dissolved aquadest in up to 100 mL.

FeCl3 0,1% solution. This solution was made from 0.1 gram of $FeCl_3$ dissolved in aquadest up to 100 mL.

Tricloroacetid Acid (TCA) 10% solution. This solution was made from 10 gram of TCA dissolved in aquades up to 100 mL.

Antioxydant Activity Using FRAP Method

The solution for FRAP methode contained 1 ml of 1500 stock solution, 1 ml phosphat buffer 0.2 M (pH 6.6), and 1 mL $K_3Fe(CN)_6$ 1%. The solution was incubated in 50ºC for 20 minute. After that, 1 mL TCA was added. The solution was then centrifuged at 3000 rpm for 10 minute. The upper solution was taken and moved to the test tube. 1 mL of aquadest and 0.5 mL of FeCl3 0.1% were also added to the same test tube. The solution was kept at a room thempetature then the absoroption was measured at 720 nm wavelenght. Oxalic acid was used for the blanko, and the FRAP level was shown by mg equivalen of ascorbic acid extract/gr extract (AAE mg/g extract).

Data Analysis

This research employed linier regression and one way ANOVA in data analysis. The analysis also used DMRT 5%, correlatioin test and linier regression if there were real differences.

RESULTS AND DISCUSSION

Total Flavonoid, Phenol and Antioxydant Activity

The flavonoids levels oin each sample were obtained from standard curve equation. The Quercetin concentration is shown by an equation line of standard curve y=0.0077x – 0.0006 and R= 0.9932 Fig. 1a. Based on the equation, acquired total of flavonoids treatment control-without phosphate buffer immersion, control of immersion in phosphate buffer, gamma rays 1000 Gy, and gamma rays 1000 Gy + EMS 1% respectively starting from 93,111, 93,157, 185,748, 108,614, and 113,080 mg of QE/G

extract Fig. 1b. This means that each gram of extract is equivalent to 93,111, 93,157, 185,748, 108,614, and 113,080 mg of quercetin.

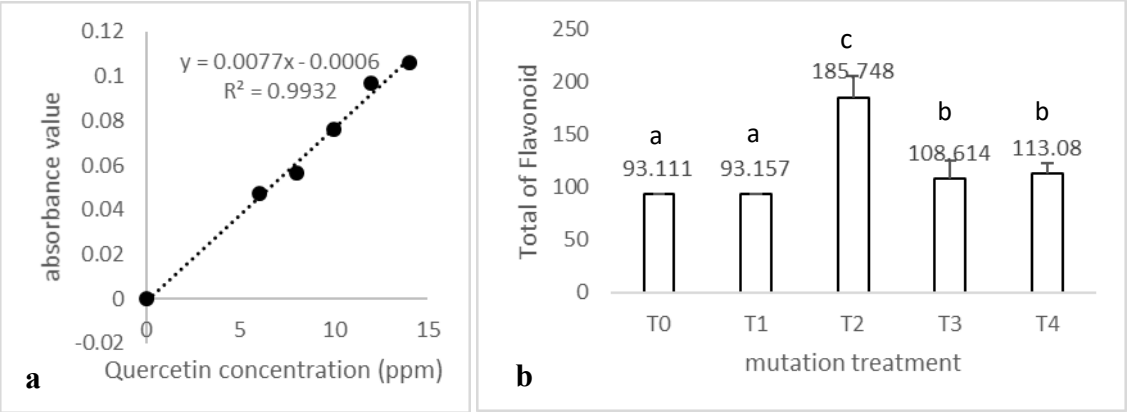

Fig. 1a. *The standard curves of Quersetin,* ***b****. Graph of total flavonoids (mg QE/g extract); Numbers followed by the same notation of DMRT (5%) means has no real difference*

Based on *Duncan Multiple Range Tests* (DMRT) 5% analysis, the result showed that the black soybeans were treatment control-without phosphate buffer immersion and control-immersion with phosphate buffer have the same response. The EMS 1% treatment and the combination gave a similar effect to the total of flavonoids levels. The gamma-ray 1000 Gy treatment produced the highest total of flavonoids levels compared to other treatments.

The Phenol levels in samples were obtained from the standard curve equation. The concentration of Gallic acid resulted in an equation line of standard curve that is y=0.0014x + 0.0073 and R= 0.9929 Fig.2a. Based on the equation, acquired the phenol levels on control treatment without phosphat buffer immersion, control treatment with phos[phat buffer immersion, 1000 Gy of Gamma rays, and gamma rays 1000 Gy + EMS 1% respectively start from 432.722, 433.421, 645.447, 570.953, dan 564.605 mg GAE/g extract Fig.2b. This means that in each gram of extract is equivalent to 432.722, 433.421, 645.447, 570.953, and 564.605 mg of Gallic acid.

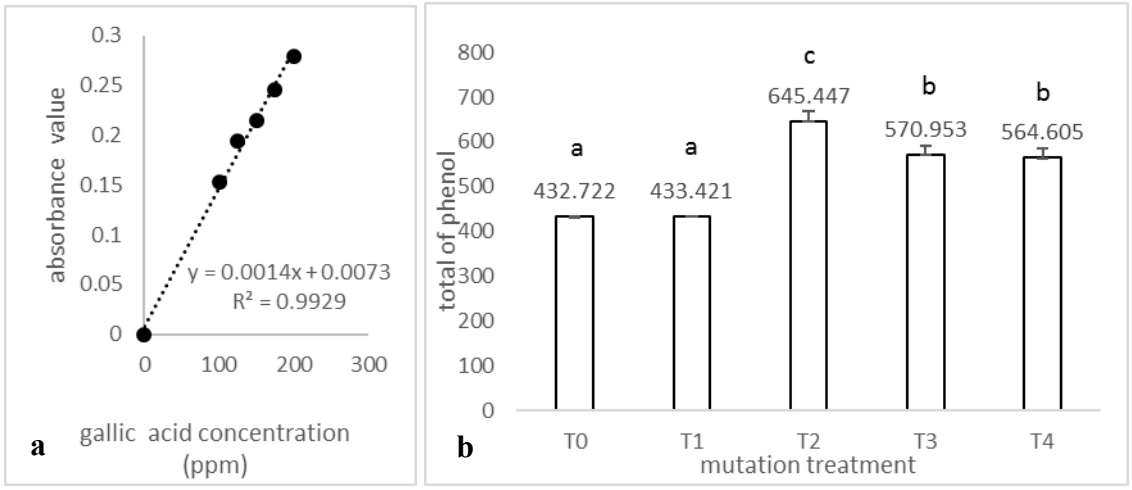

***Fig. 2a**. The standard curve of the Gallic acid, **b**. Graph of phenol content (mg of GAE/g extract); Numbers followed by the same notation of the DMRT (5%) means has no real difference*

Based on *Duncan Multiple Range Tests* (DMRT) 5% analysis, the result showed that the black soybeans were treatment control-without phosphate buffer immersion and control-immersion with phosphate have the same levels of phenol. The EMS 1% treatment and the combination gave the same phenol levels. The gamma-ray 1000 Gy treatment produced the highest total of phenol levels compared to other treatments.

The Phenol levels of samples were obtained from the standard curve equation. The FRAP is shown in MG equivalent ascorbic acid/GR extract (AAE). The concentration of ascorbic acid resulted in an equation line of standard curve that is y=0.0115x + 0.024 and R= 0.9986 Fig.3a. Based on the equation, acquired the phenol levels on control treatment without phosphat buffer immersion and control treatment with phosphat buffer immersion were obtained, 1000 Gy of Gamma rays and gamma rays 1000 Gy + EMS 1% respectivelystarting from 146.758, 146.576, 166.752, 141.012, and 153.280 mg AAE/g extract Fig.3b. This means that each gram of extract is equivalent to 146.758, 146.576, 166.752, 141.012, and 153.280 mg of ascorbic acid.

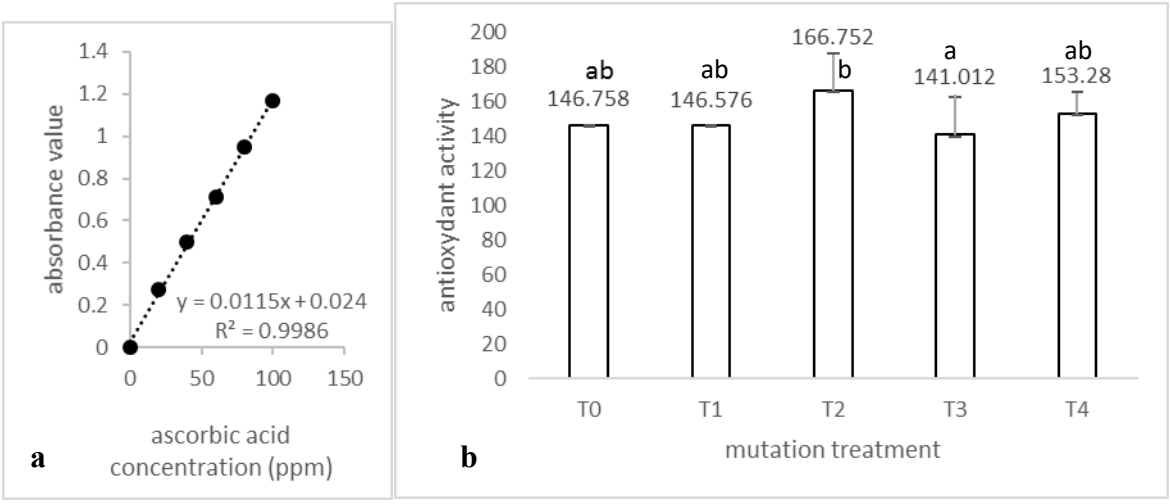

***Fig. 3a**. Ascorbic acid standard curve, **b**. Graph of antioxidant activity (AAE QE/g extract); Numbers followed by the same notation of DMRT test (5%) means has no real difference*

Based on *Duncan Multiple Range Tests* (DMRT) 5% analysis, the result showed that the Black Soybeans given the control treatment, either by immersion of buffer phosphat or not, have the same effect as the combination treatment . The EMS 1% treatment produced the lowest antioxydant activity. The gamma-ray 1000 Gy treatment produced the highest total flavonoids levels compared to other treatments..

The antioxydant activity was measured using FRAP test with ascorbic acid standard solution. TCA was added to pottasium ferrosianida precipitation. $FeCl_3$ was added to form green to blue (berlin blue) complex. Reduction power indicates potential indicators of antioxidant compounds. The reduction power was measured from the ability of the antioxidant compounds to turn Fe3 + into Fe2 + [15].

A low radiation dose can affect the growth, ploriferation, enzyme activity and the resistence to stress [16], [17]. Gamma-ray radiation interacts with atoms and molecules, causing oxidative stress i.e. excess production of reactive oxygen species [18], which is able to modify important chemical compounds in plant cells.

Gamma irradiation can lead to the release of phenolic compounds from the glycoside components and the degradation of the complex phenol compounds becomes simple, thereby increasing the total phenol [19]. Irradiation causes the radiolysis of H2O and produces free radicals i.e. hydroxyl radicals, radical hydroperoxide and hydrated electrons that can break down a glycoside bond of Procyanidin, tetramer and hexamer in plants, thereby increasing the total Phenols and Total flavonoids [20]. The use of gamma-ray irradiation is also associated with changes in the enzyme activity in the biosynthesis pathway of phenol compounds [21].

The highest results were found in 1000 Gy gamma-ray treatment. The total flavonoid was 185,748 mg QE/g extract, Phenol was 645,447 mg of GAE/g extract and antioxidant activity was 166,752 mg AAE/g of extract. It aligns with some other studies indicating that irradiation of gamma beam 20 Gy increases the accumulation of flavonoids and phenols on the callus culture of Rosemary [22]. 10 Gy dose of Gamma-ray irradiation increases the compound phenol in cinnamon [23]. Gamma-ray irradiation

significantly increases the phenolic compounds the callus culture of *F. Gummosa* at dosages 20 and 25 Gy by 36.5 and 38.9% compared to the control [24].

Huang and Mau reported that the level of phenolic compounds of irradiated samples was higher than that of unirradiated fungi [25]. Harrison reported that there was a slight increase in the total phenolic content of the irradiated almond bark extract compared to the control at a dose of 7 kGy. Dose 8 kGy increased phenolic levels in 5 soybean cultivars [26]. For samples of truffle irradiation, there was an increase in Total phenolic content at doses of Level 1 kGy and 7 kGy [19]. There is an increase in total flavonoids results of the gamma-ray irradiation on Zingiber officinale, Allium sativum and the Cesella sativa and Carulluma tubercullata [27].

Gamma-ray irradiation 1-10 kGy increases the antioxidant activity of soy beans (Ana genotypes), which is very important for health [28]. Gamma irradiation inducted in date crops at a dose of 2.5 kGy increases the antioxidant activity and total phenolic [29].

The Corellation of Total Flavonoid, Phenol and Antioxydant Activity

Total flavonoids, phenols and antioxidant activity were also analyzed using a regression correlation to determine the correlation of the three compounds. The results obtained are presented in Fig.4.

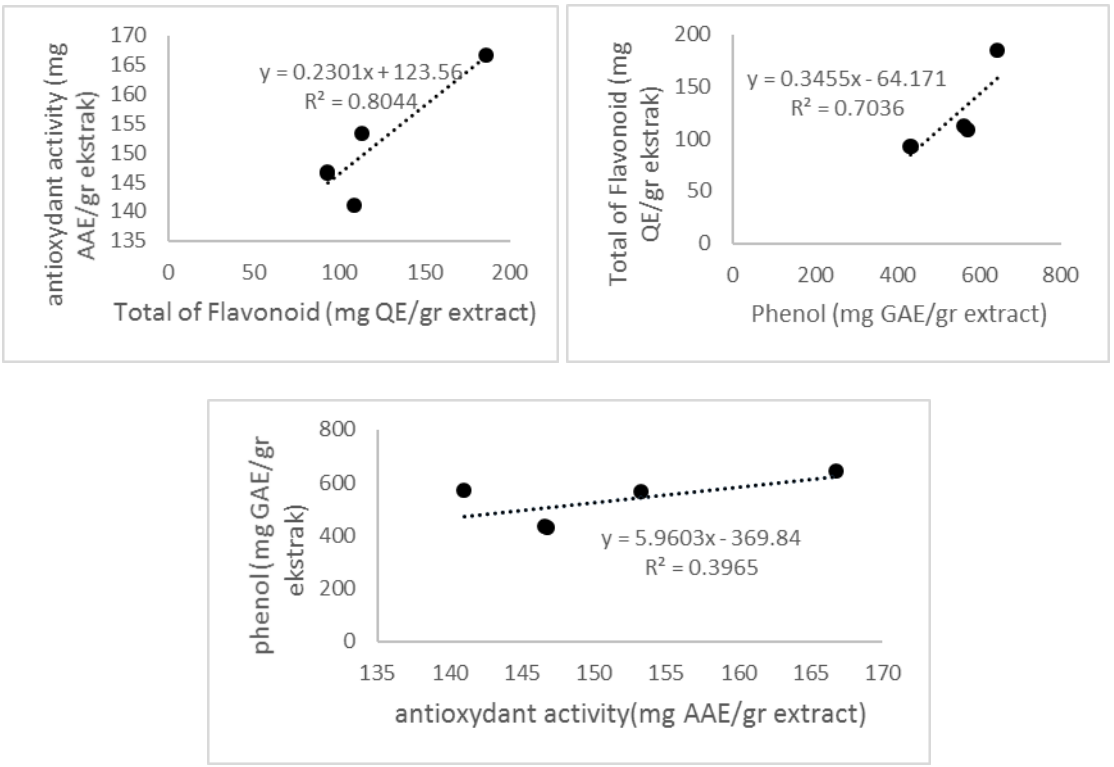

Fig. 4. *Correlation analysis of Flavonoid compounds, antioxidant activity and phenol using regression correlation*

The value of coefficient correlation (r) of flavonoids and antioxydant is 0.8044, indicating that the amount of antioxidant activity influenced by total flavonoids is high. The positive correlation (r plus value) is shown in 2 variabels in one-way test; the higher the total of flavonoids levels, the more antioxydant activity it has.

The value of flavonoids and phenol correlation was 0.7036. the number of phenol is affected by total of flavonoids. The correlation is possitive, shown by 2 variabels in one-way test which increased the total flavonoid followed by an increase in total of phenol.

Antioxydant and phenol correlation level is 0.3965; 39.65% of antioxydant activity is affected by phenol compounds, and the rest is affected by other compounds in the extract. The result of data analysis showed a possitive correlation (r plus value) between 2 variabels in one-way test. Yang [30] has reported the same result that there was a possitive correlation between phenol compounds and antioxydant activity.

Phenol compounds and flavonoid have a linear relation with antioxydant activity. Thus, the higher the levels of phenol and flavonoids, the higher the levels of antioxydant activity are [31]. Antioxidant activity is not necessarily correlated with phenol levels and total flavonoids. This is due to such factors as difference in active components, synergistic effects or antagonistic effects between active components, research conditions, and methods used to influence antioxidant activity in plants [32].

CONCLUSION

The mutagen treatment which produces total flavonoids, phenols and the highest antioxidant activity is 1000 Gy gamma ray treatment of 185,748 mg of QE/g extract, 645,447 mg of GAE/g extract, and 166,752 mg of AAE/g extract. The treatment can be used as a basis for further research on the development of black soy varieties potential as a source of antioxidants.

The limitation of this research is that it only used a spectrophotometer to determine the yield of chemical compounds (flavonoids, total phenolic content and antioxidant activity). Future research should use more modern and spesific methods such as GC-MS and in vitro mutation treatment for obtaining identical and better results.

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