Antioxidant Properties of Soybean (*Glycine max* L.) Extract and Isoflavone

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***Abstract*— Free radicals caused oxidative stress in the body, which leads to various chronic and degenerative diseases. The negative effects of free radicals can be neutralized by natural antioxidants. Soybean (*Glycine max* L.) extract contains isoflavones that have several biological activities, including antioxidants. Soybean extract (SE) antioxidant activity was evaluated compared with isoflavones (ISO). The SE was extracted in aquademineral solvent and additional lactose. SE and ISO were subject to various antioxidant activity assay such as 2,2-diphenyl-1-pycrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzo-thiazoline-6-sulfonicacid (ABTS), H_2O_2, NO scavenging activities and ferric reducing antioxidant power (FRAP) assay, following standard procedure. As a results, the IC_{50} of DPPH, ABTS, H_2O_2, NO, of SE were 246.51; 35.96; 289.41; 39.74 µg/mL respectively. While the IC_{50} value of isoflavones were 71.37; 23.57; 259.50; 11.59 µg/mL respectively. Furthermore, at the higher concentration (50 µg/mL) of SE and ISO's FRAP activity were 196.89 and 177.78 µM Fe(II)/µg. Even though the antioxidant activity of SE is lower than isoflavones, SE still has antioxidant potential. Thus, it can be used for supplement candidate.**

Keywords—soybean extract, isoflavones, antioxidant, oxidative stress

I. INTRODUCTION

Free radicals are molecules that contain unpaired electrons in atomic orbitals. Free radicals have unstable and highly reactive properties. Thus, free radicals can act as oxidizing and reducing agents. Free radical targets are all molecules in the body, especially lipids, nucleic acids, and proteins. Free radicals can cause oxidative stress by binding to molecules in cells, which causes various chronic diseases and degenerative diseases [1]. Free radicals are classified into two groups termed there are Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS include hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), peroxyl radical (•OH_2), and peroxynitrous acid (HNO_3), while the examples for RNS are nitric oxide (NO), nitrous oxide (N_2O), peroxynitrite (NO_{3-}), and nitroxyl anion [2]. H_2O_2, NO, and OH are radicals that have a role in causes oxidative stress. Free radicals can be found from the human body's metabolic processes or external exposure, such as smoking, air pollution, and industrial chemicals. To control for the negative effects of free radicals, the body needs antioxidants. Antioxidants can react with free radicals to form reactive substances that are relatively stable [3].

Different methodologies and assays are obtained to determine the antioxidant activity of samples from several origins. The principle has its origin in chemistry and has since become referred to other scientific fields such as biology, medicine, and nutrition. Summarily, it is describing a molecule's capacity to scavenge free radicals [4]. Antioxidant assays method that uses in this research is DPPH, NO, ABTS, H_2O_2, and FRAP.

The principle of H2O2 assay is the reaction between ferrous ammonium and phenanthroline inhibited by the presence of H_2O_2. Thus, it can determine the antioxidant capacity of the sample against H_2O_2 [5]. Phagocytosis, in vivo processes, cell growth regulation, intercellular signal transmission, and the synthesis of basic biological compounds all use hydrogen peroxide to produce energy. Moreover, H_2O_2 is one of the aerobic metabolism products generated and increased during exercise, diseases, and stressful situations [6].

NO is a potent signaling mediator involved in a variety of cellular processes. For example, inflammation, neurotransmission, host defense mechanisms, and vascular tone are all controlled by nitric oxide (NO) [7]. The basis of this assay is a biochemical reaction catalyzed by specific nitric oxide synthases that produce NO in biological tissues.

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For example, sodium nitroprusside reacts with oxygen in buffered saline to form nitrite ions, which can be detected using the Griess reagent [8].

The principle of DPPH assay is hydrogen donor from plant extract to bind the nitrogen group in DPPH solution. Thus, the DPPH solution becomes stable, and the color change from violet to yellow until colorless. The intensity of the color than measured by spectrophotometry to determine the antioxidant activity. The absorbance was read at 517nm due to the odd electron and the violet color of the solution. The higher absorbance indicated the higher antioxidant activity of the samples [9].

FRAP method is widely used to determine antioxidants. The principle of this method was a reduction of ferroin analog in the acidic medium. The reaction of an antioxidant compound in the sample with FRAP solution produces a complex compound [10]. The complex compound is a solution with a specific color. Then it is measured by spectrophotometry. The absorbance measurement can be related to antioxidant activity and shows how much Fe^{2+} has been reduced [11]. Based on their sources, antioxidants are classified into two categories, namely synthetic and natural antioxidants. Consumption of synthetic antioxidants in the long term could have side effects, such as skin allergies, gastrointestinal tract problems, and increasing the risk of cancer [12]. Therefore, research on natural antioxidants needs to be increased further.

Soybean (*Glycine max* L.) is one of the most popular plants for consumption due to its high nutritional value, such as vitamins A, B, C, and minerals [13]. Soybean contains many compounds such as isoflavones, saponins, phytic acid, phytosterols, trypsin inhibitors, and bioactive peptides. Soybean extract has a high content of polyphenol compounds, including isoflavones. Due to the antioxidant properties of the polyphenolic compounds, soybean extract has biological activities, such as reducing the incidence of non-communicable diseases (NCD), including cancer and cardiovascular disease [14]. Nigerian soybeans accession documented has an antioxidant activity toward DPPH, NO, FRAP, FIC, and CUPRAC methods [15]. An antioxidant is a compound that can react with free radicals. The antioxidant ability of the plant extract is due to phenolic and flavonoid compounds [16]. Several studies have shown that polyphenols, especially anthocyanins, isoflavones, and phenolic acids, are responsible for the health benefits of soybeans in general [17]. Other studies showed that soybean and isoflavones have antioxidant, antidiabetic, anticancer, and anti-inflammatory [18]. This study aims to determine the antioxidant activity of soybean extract compare with isoflavones by DPPH, ABTS, H_2O_2, NO, and FRAP assay.

Research on antioxidant activity in soybean extract has been widely conducted. However, research on Soybean Extract (SE) with GMP standards has not been widely carried out. Moreover, the comparison of the antioxidant activity of soybean extract with its compounds needs further investigation. In this study, soybean extract's (SE) antioxidant activity was investigated and comparing it with isoflavones (ISO). Thus, research was conducted to characterize the ingredient of supplement formula as immunomodulator supplement in inhibiting cytokine storm.

II. Materials and Methods

A. Samples Preparation

Soybean extracts were obtained from PT. Fathonah Amanah Sibligh Tabligh (Traditional Medicine Industry) with No. Batch 00107201055. The extraction used in this study was the maceration method with aquademineral as a solvent and additional lactose. The Soybean extract was standardized by Good Manufacturing Practice (GMP). Isoflavone (S200508) was obtained from Xi'an Sost Biotech Co., Ltd with ethanol/water solvent. The samples were diluted into DMSO 10% and store at -20 °C, and used for further assay.

B. DPPH (2,2-Diphenyl-1-picrylhydrazil) Scavenging Assay

SE and ISO were added 50 µL to the 96-well microplate. After that, add 200 µL DPPH solution (Sigma Aldrich, D9132) and incubate for 30 minutes in a dark condition. Multiskan GO Microplate Spectrophotometer, Thermo Scientific microplate reader, was utilized to measuring solution's absorbance at 517 nm. DPPH scavenging activity measures were carried out in triplicate [19] and calculated with the equation below:

$$\%\ \text{scavenging activity} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100 \quad (1)$$

C. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Reducing Activity Assay

To begin with, preparation, mix 14 mM ABTS (Sigma Aldrich, A1888) and 4.9 mM potassium persulfate (Merck, EM105091) with a volume ratio of 1:1 for 16 hours in a darkroom. Then, 5.5 mM of PBS (Phosphate Buffered Saline, pH 7.4) was added to the solution until the absorbance of the solution reached 0.70±0.02 at 745 nm. After that, 2 µL of SE and ISO and 198 µL of ABTS •+ solution were added to the 96-well microplate. After incubated for 6 minutes at 30 °C, then measuring the absorbance at 745 nm [19]. ABST reducing activity measured in triplicate and calculated with (1).

D. H_2O_2 (Hydrogen Peroxide) Scavenging Activity

SE and ISO were added 60 µL to the 96-well microplate. Then, 12 µL of 1 mM ferrous ammonium sulfate (Sigma Aldrich, 215406) was added to the well control and well blank. Next, dimethyl sulfoxide (DMSO) (Supelco, 1.02952.1000) was added 63 µL into the control well and 90 µL into the well blank. After that, 5 mM H_2O_2 was added as much as 3 µL (Merck, 1.08597.1000) to a 96-well microplate. Incubate the mixture at room temperature in dark condition for 5 minutes. After incubation, add 75 µL of 1,10-phenanthroline (Sigma Aldrich, 131377) into the 96-well microplate and incubate again for 10 minutes. The absorbance value was measured at 510 nm using a microplate reader. The experiment was carried out in triplicate [20]. H_2O_2 scavenging activity measures were carried out in triplicate and calculated with (1).

E. NO (nitric oxyde) Scavenging Activity Assay

Sodium nitroprusside 10 mM (Merck, 106541) in PBS (Gibco, 1740576) as much as 40 µl was mixed with 10 µl of

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the samples (SE and ISO). Then the mixture was incubated at room temperature for 2 hours. Then, the Griess reagent containing 1% sulfanilamide (Merck, 111799), 2% H_3PO_4 (Merck, 100573), and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich, 222488) was added as much as 100 μL into the 96-well microplate. A microplate reader was used to measuring the absorbance of the solution at 546 nm [7]. NO scavenging activity measures were carried out in triplicate and calculated with (1).

F. FRAP (Ferric Reducing-Antioxidant Power) Assay

To begin with preparation of reagent, add 1 mL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, T1253) and 1 mL of 20 mM ferric chloride hexahydrate (Merck 1.03943.0250) into 10 mL of 300 mM acetate buffer (pH 3.6). Then, 7.5 μL of samples (SE and ISO) and 142.5 μL of the FRAP reagent were inserted into the 96-well microplate. Then incubate the mixture for 6 minutes at 37 °C. Multiskan GO Microplate Spectrophotometer, Thermo Scientific microplate reader, was utilized to measuring solution's absorbance at 593 nm [21]. FRAP measured in triplicate and calculated with (1).

III. RESULT

A. ABTS Reducing Activity

The result of ABTS reducing activity in Fig.1. ABTS was obtained by reaction ABTS salt and a strong oxidizing agent such as potassium permanganate/potassium persulfate. Based on the result, the ABTS reducing activity of ISO is higher than SE. The ABTS reducing activity of ISO were 95.42%; 54.20%; 30.35%; 22.18%; 14.59%; 7.67% at final concentration. While SE were 70.07%; 35.21%; 15.53%; 4.99%; 4.26%; 3.18% respectively.

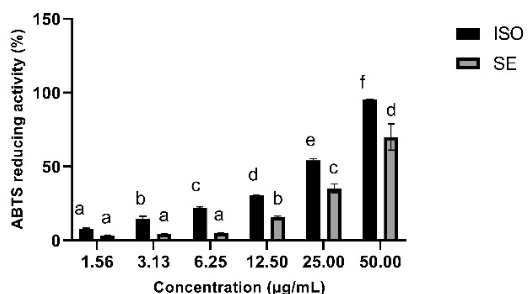

Fig. 1. ABTS scavenging activity of ISO and SE in various concentrations

*Means ± SD, n=3. Differences letters indicate the significant difference (Tukey HSD post hoc test with p <0.05)

The result of ABTS reducing activity was shown in Table I. The higher IC_{50} value indicates the lower antioxidant activity of the samples. The result indicates that ISO has higher antioxidant activity with IC_{50} 71.37 μg/mL while SE was 246.51 μg/mL. The IC_{50} value of SE toward ABTS scavenging activity is 246.51 μg/mL. SE was categorized as a moderated antioxidant activity with the IC_{50} value in 101-250 μg/mL. Furthermore, ISO was classified active antioxidant [22].

TABLE I. THE IC50 VALUE OF ABTS REDUCING ACTIVITY OF ISO AND SE

Samples	Equation	R^2	IC_{50} (μg/mL)
ISO	y = 0.5199x + 12.897	0.99	71.37
SE	y = 0.1502x + 12.974	0.99	246.51

*n=3. Equation and coefficient of regression (R^2) were obtained to calculate the IC_{50} value.

B. DPPH Scavenging Activity

The DPPH scavenging activity of ISO and SE are shown in Fig. 2. Based on Fig. 2, ISO has a higher result compared to SE. The DPPH scavenging activity of ISO and SE at 200.00 μg/mL were 64.36% and 43.00%, respectively.

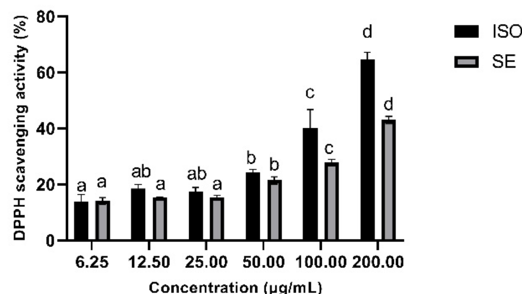

Fig. 2. DPPH scavenging activity of ISO and SE in various concentrations

*Means ± SD, n=3. Differences letters indicate the significant difference (Tukey HSD post hoc test with p <0.05)

The concentration of the sample to scavenge 50% of the DPPH free radical known as IC_{50}. The higher IC_{50} value indicates the lower antioxidant activity of the samples. Table II showed the result of the IC_{50} value of ISO and SE. ISO has high antioxidant activity with IC_{50} 23.57 μg/mL; meanwhile, SE was 35.96 μg/mL. The IC_{50} value of SE, ISO toward DPPH scavenging activity was 35.96 μg/mL, and ISO was 23.57 μg/mL. SE and ISO have categorized highly active antioxidant activity with the IC_{50} value <50 μg/mL [22].

TABLE II. THE IC50 VALUE OF DPPH SCAVENGING ACTIVITY OF ISO AND SE

Samples	Equation	R^2	IC_{50} (μg/mL)
ISO	y = 1.7582x + 8.5559	0.99	23.57
SE	y = 1.4214x - 1.1128	0.99	35.96

*n=3. Equation and coefficient of regression (R^2) were obtained to calculate the IC_{50} value.

C. FRAP Activity

The FRAP activity of ISO and SE were show in Fig. 3. An increase in absorbance indicates high reducing power. The highest FRAP activity indicates high antioxidants capacity.

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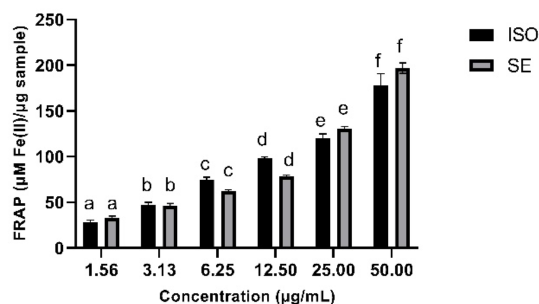

Fig. 3. FRAP activity of ISO and SE in various concentrations

*Means ± SD, n=3. Differences letters indicate the significant difference (Tukey HSD post hoc test with p <0.05)

Based on Figure 3, in the highest concentration (50 mg/mL) dan lower concentration (1.56 mg/mL) of treatment, SE showed higher antioxidant activity than ISO. However, in other concentrations of treatment, ISO showed higher antioxidant activity than SE. Based on the results, FRAP activity of SE, ISO have high antioxidant activity.

D. H_2O_2 Scavenging Activity

Determination of H_2O_2 scavenging activity in various concentrations were shown in Fig. 4. Based on Fig. 4, ISO has a little bit higher in H_2O_2 scavenging activity than SE.

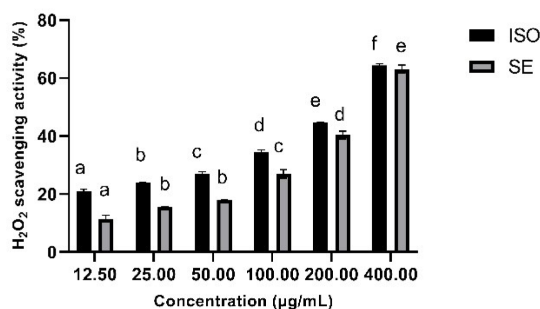

Fig. 4. H_2O_2 scavenging activity of ISO and SE in various concentrations

*Means ± SD, n=3. Differences letters indicate the significant difference (Tukey HSD post hoc test with p <0.05)

These results were also shown in Table III. The IC_{50} value of ISO was 259.51 µg/mL meanwhile, the IC_{50} value of SE was 289.41 µg/mL. The IC_{50} value indicates that ISO had has higher antioxidant activity than SE. The IC_{50} value of SE and ISO toward H_2O_2 scavenging activity was 289.41 µg/mL and 259.51 µg/mL. SE and ISO were categorized weak antioxidant activity with the IC_{50} value 250-500 µg/mL [22].

TABLE III. THE IC50 VALUE OF H_2O_2 SCAVENGING ACTIVITY OF ISO AND SE

Samples	Equation	R^2	IC_{50} (µg/mL)
ISO	y = 0.1096x + 21.558	0.99	259.51
SE	y = 0.1312x + 12.03	0.99	289.41

*n=3. Equation and coefficient of regression (R^2) were obtained to calculate the IC_{50} value.

E. NO Scavenging Activity

Based on the result shown in Figure 5, it showed that ISO has higher NO scavenging activity at the final concentration compared to SE.

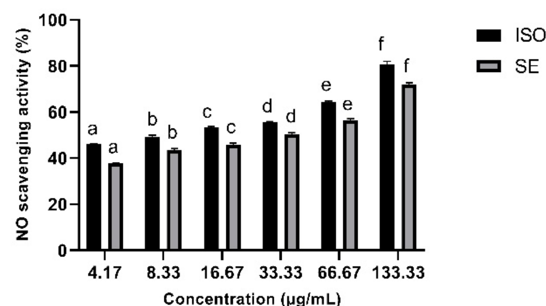

Fig 5. NO scavenging activity of ISO and SE in various concentrations

*Means ± SD, n=3. Differences letters indicate the significant difference (Tukey HSD post hoc test with p <0.05)

The result of NO scavenging activity was shown in Table IV. The higher IC_{50} value indicates the lower antioxidant activity of the samples. The SE, ISO had IC_{50} value toward NO scavenging activity 39.74 µg/mL and 11.59 µg/mL. SE and ISO have categorized highly active antioxidant activity with the IC_{50} value <50 µg/mL [22].

TABLE IV. THE IC50 VALUE OF NO SCAVENGING ACTIVITY OF ISO AND SE

Samples	Equation	R^2	IC_{50} (µg/mL)
ISO	y = 0.2546x + 47.05	0.99	11.59
SE	y = 0.2386x + 40.517	0.99	39.74

*n=3. Equation and coefficient of regression (R^2) were obtained to calculate the IC_{50} value.

IV. DISCUSSION

The ABTS assay shows that ISO has higher antioxidant activity with IC_{50} 71.37 µg/mL while SE was 246.51 µg/mL. Previous studies show that IC50 value in ABTS reducing the activity of black soybean extract is 77.39 ± 4.05 µg/mL, which is lower than IC_{50} value of daidzein with 83.34 ± 3.89 µg/mL [23]. This is due to daidzein is just one of isoflavone. Meanwhile, ISO consists of many isoflavone compounds. Thus, ISO has a lower IC_{50} compare to soybean.

Previous studies show that the DPPH scavenging activity of soybean extract 10.98 ± 0.04% [24]. In this study, ISO has higher antioxidant activity from DPPH with IC_{50} value was 71.37 µg/mL. Meanwhile, SE was 246.51 µg/mL. Therefore, the isoflavone has higher antioxidant activity than parent compounds in standard antioxidant (FRAP and TEAC) assays.

Previous studies have shown that in H_2O_2 antioxidant activity of black soybean extract has more effective than daidzein compound, which black soybean has IC_{50} 286.24 ± 11.16 (µg/mL) and daidzein compound has 366.16 ± 2.54 (µg/mL) [7]. The H_2O_2 assay in this study showed that ISO has higher antioxidant activity than SE, with IC_{50} values of ISO and SE were 259.51 µg/mLand 289.41 µg/mL,

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respectively. The results were not in line with the previous study because daidzein is just one of the isoflavones compounds. Meanwhile, ISO consists of many isoflavone compounds. Thus, ISO has better antioxidant activity compared to SE. NO scavenging activity of soybean was 64.37 ± 3.33 [24]. The results of NO scavenging assay of ISO have IC_{50} value of 11.59 μg/mL, meanwhile, SE has IC_{50} value of 39.74 μg/mL.

Other studies show the antioxidant activity of black soybean extract (BSE) compared with daidzein (standard). BSE has lower antioxidant activity in OH scavenging activity and NO scavenging activity than daidzein [2]. However, based on Asan 2019 study, BSE has high antioxidant activity in H_2O_2 scavenging activity and potentially as an antiaging agent [26].

The antioxidant method such as ABTS reducing activity (75.00 μg/mL), FRAP activity (148.89 μM Fe(II)/mg) and H_2O_2 scavenging activity (284.61 μg/mL) of EEBS show better result than daidzein (standard) [27]. These reinforce the result of the present study in antioxidant activity. Based on that, the result in the present study was better than the previous study. The SE used in this study was replenishment with lactose for GMP standardization. This might be because of the increase of antioxidant activity than the previous study.

SE has antioxidant potential. SE has lower antioxidant activity than ISO with the IC_{50} value of ABTS, reducing the activity of SE is 246.51 μg/mL. ISO is 71.37 μg/mL, DPPH scavenging activity of SE is 35.96 μg/mL, and ISO is 23.57 μg/mL, H_2O_2 scavenging activity of SE is 289.41 μg/mL, and ISO is 259.51 μg/mL, NO scavenging activity of SE is 39.74 μg/mL, and ISO is 11.59 μg/mL. However, in FRAP activity, SE has the highest antioxidant activity than ISO.

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