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FULL PAPER

Na-alginate elicitation as an alternative strategy to improve the antidiabetic potential of pigeon pea (*Cajanus cajan*) flour

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Abstract

Pigeon pea (*Cajanus cajan*) has been reported to exhibit antidiabetic activity such as hypoglycemic and hypocholesterolemic effects as well as antioxidant capacity in diabetic-hypercholesterolemia rats, alpha-glucosidase and alpha-amylase inhibitory activity, due to the bioactive compounds. Germination proved capable to increases the antidiabetic activity of pigeon pea. Elicitation has been known as a simple method for increasing the bioactive compounds and bioactivity of legumes sprouts. This study aimed to investigate the potential of Na-alginate elicitation in improving the antidiabetic activity of pigeon pea flour. The antidiabetic activity was determined by measuring the total flavonoid compounds, antioxidant activity (Trolox equivalent antioxidant activity, TEAC), alpha-amylase and alpha-glucosidase inhibitory activities. The effectiveness of Na-alginate elicitation in improving the antidiabetic activity was evaluated by comparing its antidiabetic activity to that of non-elicited pigeon pea sprout flour and non-treated pigeon pea flour. Na-alginate elicited pigeon pea sprout flour showed the highest levels of total flavonoid compounds, TEAC, as well as alpha-amylase and alpha-glucosidase inhibitory activities. Na-alginate elicitation was capable to increase the total flavonoid compounds, TEAC, alpha-amylase inhibitory activity, and alpha-glucosidase inhibitory activity of pigeon pea flour reach 107.2%, 41.7%, 237.5%, and 85.8%, respectively. It could be concluded that Na-alginate elicitation proved as an effective strategy to improve the antidiabetic potential of pigeon pea flour. These results showed positive evidence of developing legumes flour as a functional ingredient with antidiabetic potential.

1. Introduction

Diabetes mellitus is one of the four priority non-communicable diseases that have become a global health threat. Data from the International Diabetes Federation shows that people with diabetes are increasing from year to year, reaching 463 million in 2019, and is estimated to increase by 25% to 578 million people in 2030 (IDF, 2019). Maintaining blood glucose levels of DM patients in the normal range is necessary to prevent diabetes complications that induce microvascular and macrovascular diseases such as retinopathy, nephropathy, neuropathy, cardiovascular disease, and stroke (Forbes and Cooper, 2013). Therefore, diabetes management is the key strategy. One of them is through diet therapy using leguminous such as pigeon pea, lentil, chickpea, and kidney bean because they have no side effects and have the potential to prevent diabetes complications (Singhal *et al*., 2014). The bioactive compounds of legumes that contributed to antidiabetic activity among others are flavonoid compounds, alpha-amylase inhibitors, alpha-glucosidase inhibitors, and antioxidant activity (Gętek *et al*., 2014; Gothai *et al*., 2016; Ariviani *et al*., 2018). Thus, the antidiabetic potential of legumes could be evaluated by measuring the total flavonoid content, alpha-amylase inhibitory activity, alpha-glucosidase inhibitory activity as well as antioxidant activity.

Pigeon pea (*Cajanus cajan*) has been reported potentially to be developed as antidiabetic functional drinks due to the hypoglycemic and hypocholesterolemic

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effect and improving antioxidant status of diabetic-hypercholesterolemia rats (Ariviani *et al*., 2018). Uchegbu and Ishiwu (2016) reported that germinated pigeon pea flour exhibits higher levels of antioxidant activity, α-glucosidase and α-amylase inhibitory activity than that non-germinated pigeon pea flour. Elicitation is an effective strategy to increase the bioactive compounds and bioactivity of legumes sprouts rather than other conventional biotechnology (Liu *et al*., 2019). Several studies reported increases in total flavonoid compounds and antioxidant activity of legumes sprouts through elicitation such as NaCl elicitation of cowpea (Rajendra *et al*., 2019; Ariviani, Maulana, Ishartani *et al*., 2020a) and pigeon pea (Kristiani *et al*., 2019), as well as H_2O_2 elicitation of chickpea (León-López *et al*., 2020). Elicitation is also reported as an effective strategy to increase the biological activity of legumes sprouts, among others are the alpha-amylase inhibitory activity of lentil sprouts through mannitol elicitation (Świeca *et al*., 2013), ACE inhibitory activity of kidney beans sprouts through folic acid elicitation (Dueñas *et al*., 2015) and glutamic acid elicitation (Limón *et al*., 2014).

The present research aimed to evaluate the effectiveness of Na-alginate elicitation in improving the antidiabetic potential of pigeon pea (*Cajanus cajan*) flour. The effectiveness was determined by comparing the total flavonoid content, antioxidant activity, as well as alpha-amylase and alpha-glucosidase inhibitory activity of Na-alginate elicited pigeon pea sprout flour with that of non-elicited pigeon pea sprout flour and non-treated pigeon pea flour. The previous study reported that the bioactive compounds and the antioxidant activity of pigeon pea sprout prepared with Na-alginate elicitation were significantly increased along with increasing Na-alginate concentration and elicitation duration, but the germination power and the yield were decreased (Ariviani *et al*., 2021). Na-alginate concentration and elicitation duration used in this study were chosen based on Ariviani *et al*. (2021) with consideration of antioxidant capacity and the yield. Processing sprouts into flour is a simple strategy to increase the shelf life and expand its application in food processing. Elicited legume sprouts flour with the highest antioxidant retention and whiteness degree can be produced by drying at 80°C for 2 hrs (Ariviani, Mudalifah, Ishartani *et al*., 2020b). Ariviani, Lainuna and Fauza (2020) reported the potential of NaCl elicitation in improving total flavonoid, antioxidant activity, and functional properties of pigeon pea sprout flour. Alpha-amylase and alpha-glucosidase inhibitory activity, as well as total flavonoid compounds of legumes, have been studied such as in the polyphenol-rich extract of common bean (Ombra *et al*., 2018), the phenolic substance of black turtle bean and black soybean (Tan *et al*., 2017), and pigeon pea seed (Yang *et al*., 2020). To the best of our knowledge, the study on the potency of Na-alginate elicitation in the improvement of antidiabetic activity in terms of the total flavonoid content, antioxidant activity, alpha-amylase and alpha-glucosidase inhibitory activity of pigeon pea flour has not been reported.

2. Materials and methods

The materials used in this study were pigeon pea seed obtained from a local market (Surakarta, Indonesia), α-amylase from human salivary type XIII-A, α-glucosidase from *Saccharomyces cerevisiae* type I, DNS (3,5-dinitrosalicylic acid), pNPG (4-Nitrophenyl α-D-glucopyranoside), quercetin, potassium persulfate, ABTS (2,2'Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were obtained from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid, Aluminum chloride, Sodium acetate, sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, Potassium dihydrogen phosphate, sodium hydroxide, DMSO (Dimethyl sulfoxide), and sodium carbonate were obtained from Merck Millipore (Darmstadt, Germany). Potato starch was obtained from Riedel-de Haen (Holzheim, Germany). Acarbose was produced by OGB Dexa (Tangerang, Indonesia). All reagents used in this study were analytical grade.

2.1 Pigeon pea sprout preparation

Pigeon pea sprouts were prepared as previously described by Ariviani *et al*. (2021) with slight modification. Briefly, the pigeon pea seeds were sorted and then rinsed three times using distilled water. Furthermore, the seeds were submerged in 250 ppm Na-alginate solution or distilled water (without elicitation) with a ratio of 1:3 w/v for 16 hrs, followed by germination for 60 hrs.

2.2 Flour preparation

Flour preparation was conducted according to Ariviani, Lainuna and Fauza (2020). Briefly, the pigeon pea seed, pigeon pea sprout (without elicitation), and Na-alginate elicited pigeon pea sprout were dehulled and dried in a cabinet dryer (Xingtai XTDQ-101-4, Jiangsu, China) at 80°C until the moisture content reaches 8±2% or for about 2 hrs for the sprouts and 30 mins for the seed. The dried materials were powdered and sieved using a 60-mesh sieve (Virsair CF812-01, Indonesia).

2.3 Total flavonoid compounds and antioxidant activity determinations

Initially, Na-alginate elicited and non-elicited pigeon

pea sprout flours, as well as non-treated pigeon pea flour, were extracted using double distilled water with the ratio of 1:20 w/v in a water bath shaker (SWB 20, Fisher Scientific Haake, Germany) at 200 rpm, 50°C for 1 hr. Each extract was centrifuge using PLC-05 Centrifuge (Gemmy, Taiwan) at 10000 rpm for 10 mins to obtain clear supernatant, which was collected in dark bottles and stored at 10°C until the analysis was conducted. The total flavonoid compounds of flour extracts were analyzed using the aluminium complexation reaction method in the absence of $NaNO_2$ (Pękal and Pyrzynska, 2014). The total flavonoid compounds were expressed as Quercetin equivalent (QE) (mM QE/100 g flour dry weight). The flavonoid standard curve was established using 100 – 300 µM quercetin solution. Antioxidant activity evaluation was conducted by measuring the radical scavenging activity (RSA) using the ABTS•+ free radical method (Re *et al*., 1999), and expressed as Trolox equivalent antioxidant capacity (TEAC) (mM TEAC/100 g flour dry weight). The RSA standard curve was established using 40 – 240 µM Trolox solution.

2.4 Analysis of alpha-amylase and alpha-glucosidase inhibitory activity

The flour samples which include non-treated pigeon pea flour, non-elicited and Na-alginate elicited pigeon pea sprout flour was extracted using double distilled water as was done on flavonoid and antioxidant activity evaluation. Before being analyzed, the clear extracts were concentrated using a rotary evaporator (Buchi Rotavapor, Switzerland) at 50°C, 500 mBar. The concentrates were freeze-dried (Edwards Modulyo, UK) at (-) 40°C and 0.1 Torr for 72 hrs to produce dried extracts. These extracts were collected, then vacuum-packed, and stored at 4°C until further analysis. Acarbose was used as a positive control for the alpha-amylase and alpha-glucosidase inhibitory evaluation. Acarbose is a diabetic drug with a mechanism of action through a competitive and reversible inhibitor of human carbohydrates hydrolyzing enzymes, namely pancreatic alpha-amylase and membrane-bound intestinal alpha-glucoside hydrolase (McIver and Tripp, 2021).

The alpha-amylase inhibitory activity was analyzed using the DNS method (Yang *et al*., 2020) with slight modification. Briefly, 500 µL of 0.02 M phosphate buffer saline (pH 6.9, containing 6 mM NaCl) was added with 1 mL of human salivary alpha-amylase (0.25 U/mL) and 1 mL of 1 mg/mL flour extracts or 1 mg/mL acarbose. The mixtures were incubated at 37°C for 10 min, and 580 µL of 1% w/v potato starch solution was added, further incubation at 37°C for 15 mins. 1 mL DNS (3,5-dinitrosalicylic acid) reagent was added to stop the reaction and directly placed in a water bath (SWB 20, Fisher Scientific Haake, Germany) at 100°C for 5 mins, cooling to ambient temperature and diluted (2 times), then the absorbance was measured at 540 nm using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The control (without either extract or acarbose additions) represents 100% enzyme activity. The alpha-amylase inhibitory activity was determined using following formula: % AIA = ((abs control - abs sample)/abs control) × 100%. The alpha-amylase inhibitory activity of flour samples was expressed as % AIA/mg flour dry weight.

The alpha-glucosidase inhibitory activity test was carried out using the pNPG hydrolysis method (Guo *et al*., 2019) with slight modification. Briefly, 20 µL of 1 mg/mL sample solutions (the flour extracts or acarbose) in DMSO or 20 µL DMSO (as blank) were added with 30 µL of alpha-glucosidase from Saccharomyces cerevisiae type I (2 U/mL in 0.1M potassium phosphate buffer, pH 6.8). The mixture was added in 650 µL of 0.1M potassium phosphate buffer (pH 6.8), incubated at 37°C for 5 mins, and further added to 150 µL of 10 mM p-NPG as substrate, followed by incubation at 37°C for 30 mins. The reaction was stopped by the addition of 650 µL of 1 M Na2CO3. The absorbance of the final mixture was measured at 405 nm using a UV -VIS spectrophotometer (UV-1800, Shimadzu, Japan). The alpha-glucosidase inhibitory activity was determined with the following formula: % GIA = ((abs blank- abs sample)/ abs blank) × 100%. The alpha-glucosidase inhibitory activity of samples was expressed as % GIA/ mg flour dry weight.

2.5 Statistical analysis

The Data was expressed as means ± standard deviation from three replication. The IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA) program with Analysis of Variance (ANOVA) at the significant level of p-value<0.05 was used for data analysis. Duncan's Multiple Range Test (DMRT) with a similar significant level (p-value<0.05) was used to evaluate the significant difference between means.

3. Results and discussion

3.1 Total Flavonoid Compounds (TFC)

Flavonoid compounds exhibit antidiabetic activity through several mechanisms, among others are inhibiting alpha-amylase and alpha-glucoside activity through enzyme-flavonoids complexation (Yuan *et al*., 2014; Proenc *et al*., 2017), radical scavenging activity, increasing insulin sensitivity and Glute 4 transporter, as well as anti-inflammatory activity (Vinayagam and Xu, 2015). Total flavonoid compounds (mM QE/100g flour dry weight) of Na-alginate elicited pigeon pea sprout

flour was significantly higher than that of non-elicited pigeon pea sprout flour and non-treated pigeon pea flour, i.e., 46.19, 34.31, and 22.29, respectively for Na-alginate elicited and non-elicited pigeon pea sprout flours, non-treated pigeon pea flour (Table 1). During germination, several enzymes which induce secondary metabolite biosynthesis, including flavonoid biosynthesis, are activated, therefore pigeon pea sprout flour exhibited higher total flavonoid compounds than non-treated pigeon pea flour (Ariviani, Lainuna and Fauza, 2020). Elicitation is an expeditious technique to increase antioxidant compounds, including flavonoid compounds of legumes sprouts as the response to elicitation stress (Rajendra *et al*., 2019).

The results in Table 1 indicated that the TFC improvement of pigeon pea flour due to germination (without elicitation) was significantly lower than that of germination with Na-alginate elicitation, i.e. reach 53.9% for germination and 107.2% for germination with Na-alginate elicitation. Ariviani, Lainuna and Fauza (2020) reported similar results. The TFC improvement of pigeon pea flour due to germination with NaCl elicitation was significantly higher than germination without elicitation. The TFC improvement of pigeon pea flour due to germination with NaCl elicitation reached 41.8%. It indicated that Na-alginate elicitation produces higher TFC improvement of pigeon pea flour than NaCl elicitation. It was related to the higher stress levels induced by Na-alginate elicitation than NaCl elicitation. Ariviani *et al*. (2021) stated that the higher stress level induces higher secondary metabolite biosynthesis, such as phenolic and flavonoid compounds in elicited legumes sprouts. The present research results suggested that Na-alginate elicitation is a promising strategy to improve the antidiabetic potential of pigeon pea flour in terms of TFC.

3.2 Antioxidant activity (TEAC)

The radical scavenging activity (RSA) of Na-alginate elicited and non-elicited pigeon pea sprout flour, as well as non-treated pigeon pea flour, were presented in Table 1. Na-alginate elicitation produces pigeon pea flour with the highest TEAC, followed by germination without elicitation, and the lowest in the non-treated one. A similar data phenomenon was observed in TFC (Table 1). Flavonoid compounds exhibit the ability to reduce free radicals by quenching free radicals directly via proton transfer from the A and/or B rings of the flavonoids and forming less active flavonoid radicals. The radical scavenging ability of flavonoid compounds is related to the flavonoid structure, such as the presence, position, and modification of the hydroxyl groups on rings A and B (Mierziak *et al*., 2014). Previous study reported the strong correlation between the TFC and the TEAC of legumes, such as in lentils ($r = 0.8783$, $p<0,01$) (Alshikh *et al*., 2015), faba bean ($r = 0.884$, $p<0.01$) (Lu *et al*., 2018), and cowpea ($r = 0.962$, $p<0,01$) (Rajendra *et al*., 2019).

Increases in antioxidant activity have been observed in legumes sprouts due to the initiation of various biochemical reactions during germination (Sharma and Singh, 2018). Elicitors used as stress inducers during elicitation act as triggers in the alteration of cell structure and cellular molecules metabolism, and promote the reprogramming of plant defense signaling pathways (Ampofo *et al*., 2020), one of them by increasing antioxidant activity (TEAC) as observed in chickpea sprout (León-López *et al*., 2020), cowpea sprout (Rajendra *et al*., 2019).

Germination treatment is capable to enhance the TEAC of pigeon pea flour by 34.2%, whereas the Na-alginate elicitation before germination results in elevated TEAC by 41.7%. It indicated that Na-alginate elicitation efficiently improves the antidiabetic potential of pigeon pea flour, especially in the radical scavenging activity. One of the risk factors for diabetes is stress oxidative. Antioxidants with the mechanism of radical scavenging will reduce oxidative stress by terminating the radical chain reaction. The study by Gudise *et al*. (2019) showed that the *Matelea denticulate* leaf extract with the highest radical scavenging activity showed the highest antidiabetic activity.

3.3. Alpha-amylase inhibitory activity

All of the flour samples exhibited alpha-amylase inhibitor activity (Table 2). The alpha-amylase inhibitory activity of non-treated pigeon pea flour, non-elicited and Na-alginate elicited pigeon pea sprout flours were 1.89, 3.06, and 6.38 %AIA/mg flour dry weight, respectively.

Table 1. Total flavonoid compounds and antioxidant activity of Na-alginate and non-elicited pigeon pea sprout, non-treated pigeon pea flour

Flour Samples	Total Flavonoid compounds (mM QE/100 g flour dry weight)	Trolox equivalent antioxidant capacity (mM TEAC/100 g flour dry weight)
Non-treated pigeon pea flour	22.29±1.62[a]	11095.20±316.94[a]
Non-elicited pigeon pea sprout flour	34.31±2.04[b]	14892.80±651.87[b]
Na-alginate elicited pigeon pea flour	46.19±3.81[c]	15724.37±569.90[b]

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (p-value<0.05).

Table 2. Alpha-amylase and alpha-glucosidase inhibitory activity of Na-alginate and non-elicited pigeon pea sprout, non-treated pigeon pea flour

Flour Samples	Alpha-amylase inhibitory activity	Alpha-glucosidase inhibitory activity
Non-treated pigeon pea flour	1.89±0.01[a]	225.74±18.29[a]
Non-elicited pigeon pea sprout flour	3.06±0.06[b]	279.78±4.06[b]
Na-alginate elicited pigeon pea flour	6.38±0.45[c]	419.52±20.40[c]
Acarbose (positive control)	55.99±3.65[d*]	1799.92±115.72[d**]

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (p-value<0.05).

* % AIA/mg sample wet weight. ** % GIA/mg sample wet weight

These activities reach 3.4%, 5.5%, and 11.4% of the inhibitory activity of the diabetic drug "acarbose". Alpha -amylase inhibitory activity of the three flour samples was much lower than the acarbose inhibitory activity. It was related to the units used to express the inhibitory activity of the flour samples, which was expressed per mg flour dry weight. This unit is expected to give more representative information of the serving size that must be consumed to provide antidiabetic activity, especially the alpha-amylase inhibitory activity which is equivalent to 1 tablet (50 mg) of the diabetic drug "acarbose". Although, as consequence, the inhibitory activity seems too poor when compared to the acarbose activity. The serving size of Na-alginate elicited pigeon pea sprout flour of 783.7 mg equal to 1 tablet of acarbose in terms of alpha-amylase inhibitory activity.

Table 2 indicated that the alpha-amylase inhibitory activity of pigeon pea flour could be significantly improved through germination. The inhibitory activity of the pigeon sprout flour was 62% higher than that of non-treated pigeon pea flour. Uchegbu and Ishiwu (2016) who studied the effect of germination on antidiabetic activity, especially the alpha-amylase inhibitory activity of pigeon pea, reported similar results. The improvement of alpha-amylase inhibitory activity due to the germination was related to the secondary metabolite produced during the germination process. It was supported by the TFC data in Table 1. The pigeon pea sprout flour has higher TFC rather than non-treated pigeon pea flour. Flavonoid compounds exhibit non-competitive alpha-amylase inhibitory activity through the formation of a complex between the flavonoids and the enzyme (Yuan *et al*., 2014).

Improvement of alpha-amylase inhibitory activity of pigeon pea flour promoted by the treatment of Na-alginate elicitation prior to germination reach 237.5%, from 1.89 % AIA/mg flour dry weight (non-treated pigeon pea flour) to 6.38 % AIA/mg flour dry weight (Na-alginate elicited pigeon pea sprout flour). This result was related to the highest TFC and TEAC induced by the treatment (Table 1). It indicated that Na-alginate elicitation could be an alternative strategy to enhance the antidiabetic potential of pigeon pea flour. Yuan *et al.* (2014) reported that alpha-amylase is one of the key enzymes in the gastrointestinal tract that catalyzes the initial step in carbohydrate hydrolysis. The inhibition of the alpha-amylase activity is able to effectively control the increase of postprandial blood glucose levels.

3.4 Alpha-glucosidase inhibitory activity

Inhibition of alpha-glucosidase activity delays the digestion and absorption of carbohydrates intake by preventing the hydrolysis of maltose, maltotriose, and oligosaccharide, into glucose, therefore suppressing the blood glucose levels elevation (Proenc *et al*., 2017). The alpha-glucosidase inhibitory activity of non-treated pigeon pea flour, pigeon pea sprout flour, and Na-alginate elicited pigeon pea sprout flour presented in Table 2 indicated that Na-alginate elicitation before germination is effective in increasing the antidiabetic potential of pigeon pea flour. The enhancement of alpha-glucosidase inhibitory activity by germination without and with Na-alginate elicitation were 23.9% and 85.8% respectively, i.e. 225.74 % GIA/mg flour dry weight in non-treated pigeon pea flour, 279.78 % GIA/mg flour dry weight in pigeon pea sprout flour, and 419.52 % GIA/mg flour dry weight in Na-alginate elicited pigeon pea sprout flour. The potential of Na-alginate elicitation in the improvement of alpha-glucosidase activity of pigeon pea flour has been related to the capability of increasing the TFC level. Flavonoid compounds widely distributed in plants are notable to play a role in lowering blood glucose through the inhibition of alpha-glucosidase (Pereira *et al*., 2011; He *et al*., 2019). The alpha-glucosidase inhibitory mechanism of flavonoid compounds by flavonoid-enzyme complexation through competitive, non-competitive, and mixed competitive and non-competitive inhibition (Proenc *et al*., 2017).

4. Conclusion

Both germination treatments with and without Na-alginate elicitation proved capable to elevated the antidiabetic potential of pigeon pea flour through the enhancement of the TFC, TEAC, as well as alpha-amylase and alpha-glucosidase inhibitory activity. The

enhancement of the antidiabetic activity triggered by Na-alginate elicitation was significantly higher than that promoted by germination without elicitation. It could be concluded that Na-alginate elicitation can be considered an effective technique to increase the antidiabetic potential of legumes flour.

Conflict of interest

The authors declare no conflict of interest.

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