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Original Research Article

Alpha-Amylase Inhibitory Activity of Extract Combination of *Morinda citrifolia* L. Fruit, *Trigonella foenum*-graecum and *Nigella sativa* L. Seeds Using *In vitro* and *In Vivo* Assay

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ABSTRACT

The poly-herbal formulation is being evaluated for effective diabetes management. However, only a few formulations have made significant inroads as treatment alternatives. This research was carried out to determine the *in vitro* and *in vivo* alpha-amylase inhibitory activity of extract combination of *Morinda citrifolia* L fruits, *Trigonella foenum*-graecum and *Nigella sativa* L. seeds, because each extract was known to have antidiabetic properties. *In vitro* alpha amylase activity was carried out using dinitrosalicylic acid (DNS) assay while the *in vivo* alpha amylase activity was performed using experimental rat model. Twenty-four mice (*Mus musculus* L.) were randomly divided into six groups. Group 1 served as the control and was given distilled water only, while group II was induced with alloxan (40 mg/kg *i.p*.) only. Groups III, IV, V and VI rats were induced with alloxan, then after 48 h of induction, the rats were given acarbose, dose 1 (70:70:280 mg/kg BW): dose 2 (140:70:140 mg/kg BW) and dose 3 (70:140:140 mg/kg BW), respectively. The three combination of extracts showed inhibitory alpha-amylase activity, with combination of dose 2 as the best potential inhibition. The combination of dose 2 has an IC_{50} value of 35.70 ± 1.33 µg/mL and the percentage reduction in the average blood glucose levels at the 30th, 60th, 120th minute were shown to be 11.98%, 25.85%, and 34.80%, respectively.

Keywords: Blood glucose level, Diabetes mellitus, IC_{50}, *Morinda citrifolia* L., *Nigella sativa* L., *Trigonella foenum-graecum* L.

Introduction

Maintaining glycemic control in Diabetes Mellitus (DM) is necessary to prevent many health complications, mortality, and morbidity.[1] There are various pharmacological approaches used in DM therapy such as delay the digestion and absorption of carbohydrates in the gastrointestinal system by inhibiting alpha-amylase and alpha-glucosidase activity.[2] Alpha-amylase will converts polysaccharides in the mouth and intestines into the disaccharide molecule, then the reaction will be continued by alpha-glucosidase to produce glucose units that will be absorbed into the body.[3] This inhibition can lessen postprandial hyperglycemia and prevent diabetic complications.[4]
There are many studies about the alpha-amylase inhibitory activity of plants and their bioactive compounds *in vitro*, but few studies confirm these findings in rodents and very few in humans.[5] *In vivo* and *in vitro* studies may not always be correlated, but *in vitro* data can be used to determine the activity of compounds before use in *in vivo* studies to determine physiological and pharmacological effects in animals.[6]
Based on the previous study, the ethanol extract of Fenugreek seeds (FS), Morinda Fruit (MS), and Black Cumin Seeds (BCS) were reported to have antidiabetic activity, [7-9] but there were no reports regarding the inhibitory activity of alpha-amylase from the combined ethanol extract of (FS), (MF) and (BCS) either *in vitro* or *in vivo*. Ethanol extract of *Trigonella foenum-graecum* seeds or Fenugreek seeds (FS) has the IC_{50} value of inhibition of alpha-amylase as 1.87 mg/mL[10] and the *in vivo* studies showed significant blood glucose-lowering capacity in alloxan-induced diabetic rats at different doses (0.1, 0.5, 1, and 2 g/kg).[9] Ethanol extract of *Morinda citrifolia* L. fruit or *Morinda Fruit* (MF) in unhydrolyzed, hydrolyzed and fermented products were given the IC_{50} value as 18.53, 5.88, and 2.57 mg/mL[11] and the *in vivo* studies showed antidiabetic activity in alloxan-induced diabetic rats and OGTT at doses 0.5 and 1 g/kg.[10] Meanwhile, ethanol extract of *Nigella sativa* L. seeds or Black Cumin Seeds (BCS) can play an antihyperglycemic role at different doses (0.3, 0.5, 0.81, and 2 g/kg),[12] as an agonist PPAR-gamma, induce insulin-like effects on skeletal muscle cells and adiposites[13] and the *in vitro* studies of ethanol extract of BCS showed inhibition value between 11.55 ± 0.78% in concentration 62.5 µg/mL.[14]
Management of postprandial blood glucose levels in diabetic patients are usually given when a high HbA1c levels, so it is generally used more than one type of therapy to normalize metabolism and prevent complications. Patients are usually given a combination of drugs such as metformin and acarbosa, but the risk of side effects is greater.[15]
Poly-herbal formulations are being evaluated for effective diabetes management with few side effects, the use of more than one herb aims to control more than one disease-causing factor. However, only a few formulations have made significant inroads into alternative treatments. Therefore, we combined the ethanolic extracts of FS, MS, and BCS to investigate the antidiabetic potential of a new poly-herbal formulation with the mechanism of alpha-amylase inhibitory activity with *in vitro* and *in vivo* studies.

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Materials and Methods

Reagents
The solvents and reagents used are 70% of ethanol, alpha-amylase (Sigma Aldrich Cat. No A3176), potato starch/Soluble starch (Merck), 3,5-dinitrosalicylic acid (Sigma Aldrich), Potassium dihydrogen phosphate (Merck), Sodium hydroxide (Merck), Sodium Carbonate (Merck), Demineralization, Phenols (Merck), Potassium Sodium Tartrate Tetrahydrate (Merck), Sodium Metabisulfite (Merck), Alloxan (Sigma Aldrich), Acarbose, and 2% of Pulvis Gummi Arabicum.

Preparation of extracts
The plant materials of FS, MF, and BCS were collected from Lembang, West Java, Indonesia during May 2018. Those plants were authenticated at Plant Taxonomy Laboratory, Department of Biological Science, Universitas Padjadjaran with No.679/HB/05/2018. Scientific names of the plant species were verified using on line sources (e.g., The Plantlist, 2018).[16] The dried of FS, MF, and BCS from the sorting and chopping process were weighed (500 g) and extracted by maceration for 72 hours using 3 L ethanol (70%). Then the product of maceration is concentrated into a liquid extract using a rotary evaporator at a temperature of 40-50°C and then evaporated until it becomes a thick extract with constant weight. The combined extract was obtained by mixing the ethanol extract of FS, MF, and BCS into three comparison doses as indicated in Table 1.

Table 1: The dose combination of each extract of FS, MS and BCS

Dose	FS (mg)	MF (mg)	BCS (mg)	Final combination dose (mg/kg BW) FS:MF:BCS
1	70	70	280	70 : 70 : 280
2	140	70	140	140 : 70 : 140
3	70	140	140	70 : 140 : 140

Experimental animals
Swiss Webster mice (*Mus musculus* L), male, minimum age of 8-10 weeks, in the range of 20 – 30 g in body weight, physically normal and healthy. The animals were supplied by Eyckman Pharmacology Laboratory RSHS Bandung, West Java, Indonesia. Ethical clearance was granted by the Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran with number 665/UN6.KEP/EC/2019.

In vitro studies
The assay method was modified from Chavan & Wadatkar (2014).[17] The optimization of substrate was carried out on six variants of starch concentrations ranging from 0.5 mg/mL to 15 mg/mL. A 200 µL of starch, 200 µL of 2 U/mL alpha-amylase solution were homogenously mixed into test tube. After incubated at 37°C for 3 min, 200 µL of colour reagent (dinitrosalicilic acid) and boiled for 10 minutes in a water bath at 85-95°C. The mixture was cooled to ambient temperature and diluted with 5 mL of distilled water, and the absorbance was measured at a wavelength of 540 nm using a UV-Visible spectrophotometer.
Furthermore, alpha-amylase inhibition assay was performed using the DNS Bernfeld (1995)[18] from Sigma-Aldrich that was modified by Wickramaratne *et al* (2016).[19] Each Dose of sample extract were dissolved in phosphate buffer ($(Na_2HPO_4/NaH_2PO_4$ (0.02 M), NaCl (0.006 M) at pH 6.9) to give five concentrations that are 15.625, 31.25, 62.5, 125 and 250 µg/mL. A volume of 200 µL alpha-amylase solution (2 U/mL) was mixed with 200 µL sample extract and was incubated for 10 minutes at 37°C. After that 200 µL of the optimized starch solution was added to each tube and incubated for 3 minutes at 37°C. The reaction was stopped by adding 200 µL of DNS reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) and boiled for 10 minutes in a water bath at 85-95°C. The mixture was cooled to ambient temperature and diluted with 5 mL of distilled water, and the absorbance was measured at a wavelength of 540 nm using a UV-Visible spectrophotometer. The blank was prepared with a similar procedure in a sample extract without enzyme, the enzyme was replaced by a phosphate buffer. The positive control (acarbose) was prepared with a similar reaction in a sample extract as mention above. Each measurement was carried out three times. The percent inhibition and of alpha-amylase activity was calculated by the following equation:

Inhibition (%) = [(Ac-Acb) - (As-Asb)/(Ac-Acb)] x 100%

Where Ac is the absorbance of control (substrate + enzyme, without sample); Acb is the absorbance of control blank (substrate + buffer, without enzyme and sample); As is the absorbance of sample; and Asb is the absorbance of sample blank. IC_{50} was calculated using a linear equation where the concentration of the sample as the x-axis and % inhibition as the y-axis. From the equation y = ax + b, IC_{50} can be calculated by using the formula:

$$IC_{50} = [(50-b)/a].$$

In vivo studies
Twenty-four mice (*Mus musculus* L.) were randomly divided into six groups. Acclimatization was conducted in one week. Before treatment, mice fasted (not eating but given water) for 18 h. DM model was made by inducing mice with 200 mg/kg BW of alloxan monohydrate through the intraperitoneal route to each group except the normal group[20] After 48 h, blood glucose level was measured and and mice with blood glucose concentration above 200 mg/dL were considered diabetic.
In vivo assay was performed by oral starch tolerance test, the assay method was modified from Poovitha and Parani (2015).[21] Tests were carried out on 5 groups of mice that had been made by DM models and one normal group. Before testing, each group fasted for 18 h, then weighed and their blood glucose level was calculated before the treatment. Furthermore, each group was given the following treatment:

1. Normal control (CN): normal mice, distilled water
2. Negative control (C(-)): diabetic mice, distilled water
3. Positive control (C(+)): diabetic mice, PGA 2% + acarbose 6.5 mg/kg BW
4. Treatment group 1 (D1): diabetic mice, PGA 2% + Dose 1
5. Treatment group 2 (D2): diabetic mice, PGA 2% + Dose 2
6. Treatment group 3 (D3): diabetic mice, PGA 2% + Dose 3

Each preparation was given orally. Blood glucose measurements were carried out 10 minutes after treatment (t = 0). Then, 3 g/kg BW of starch solution was administered to all mice and the measurements of blood glucose levels were carried out again at 30^{th}, 60^{th} and 120^{th} minute using a glucometer and a test strip (Accu-Chek Active, Roche Diagnostics GmbH, D-68298 Germany).

Statistical analysis
Results are expressed as mean ± standard error (SE) and differences between means were statistically analyzed using one-way analysis of variance (ANOVA) followed by a Student Newman Keuls (SNK) test at a significant level (α) 0.05.

Results and Discussion

Inhibition of α-amylase activity using In Vitro Assay
The alpha-amylase inhibition assay was initiated by involving the optimization of a substrate. Optimization of the substrate and enzyme concentration is performed to determine the optimum substrate concentration for the test by 2 U/mL alpha-amylase enzyme. The

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result of optimum substrate concentration was 5 mg/mL. At this concentration, when the substrate is made even greater, the absorbance does not increase because the enzyme has reached its saturation point (all active sites are already occupied by the substrate). The results is shown in Figure 1.

Inhibition of α-amylase activity was found to be dependent on concentration, the IC_{50} of each sample was determined as the inhibitor concentration required to inhibit 50% of alpha-amylase activity under the described experimental conditions, the result can be seen in Table 2. The IC_{50} value obtained from Acarbose was 83.07 ± 2.16 µg/mL. Meanwhile, the dose combination 2 has the best inhibitory activity of alpha-amylase with IC_{50} value of 35.7 ± 1.33 µg/mL. This may be due to the phytochemical composition of the dose which has a higher amount of FS compared to combinations 1 and 3.[22] In the study of Musfiroh *et al* (2019),[23] the ethanol extract of FS, MS and BCS contain alkaloids, flavonoids, monoterpenes and sesquiterpenes.[23,18] Polyphenols, especially flavonoids have the important property of inhibiting alpha-amylase and alpha-glucosidase.[10] Flavonoids have therapeutic properties that can modulate the number of cell signaling pathways and affect carbohydrate digestion, fat deposition, and the rate of insulin release or glucose uptake in insulin responsive tissue. The interaction between flavonoids and alpha-amylase shows that the inhibitory potential correlates with the number of hydroxyl groups in the *β*-ring of the flavonoid skeleton.[24] Flavonoids can prevent complications or progression of DM by clearing excessive free radicals, breaking the chain of free radical reactions, binding of metal ions (chelating) and blocking the polyol pathway by inhibiting the enzyme aldose reductase. Flavonoids also have an inhibitory effect on the α-glucosidase enzyme through hydroxylation bonds and substitution in the *β*-ring. Flavonoids are strong inhibitors of the α-amylase enzyme that function in the breakdown of carbohydrates. The inhibitory power of this enzyme causes the process of breaking down and absorption of carbohydrates to be disrupted, so that blood sugar levels can be reduced.[25] Previous research reported that the ethanol extract of FS has total phenolic content (TPC) of 81.55 mg GAE/g,[26] which was higher than MF (79.57±0.14 mg GAE/100g)[27] and BCS (4.258 ± 0.2 mg GAE/g).[28]

Blood Glucose Level Reduction using In Vivo Assay

The *in vivo* results were made into relative blood glucose levels and the percentage of the reduction levels was also calculated. Based on Figure 2, it can be seen that the blood glucose levels of all groups showed a different peak point, this shows the differences in digestibility of starch in each treatment group due to alpha-amylase inhibition in mice. In the negative (C(-)), positive (C(+)) and treatment 3 (D3) groups, blood glucose levels peaked at the 60th minute. This occurred because in the body of diabetic mice there are abnormalities of the homeostatic mechanism due to alloxan induction, where administration of alloxan can cause free radical formation and damage cell membrane permeability resulting in damage of pancreatic beta cells and cause dysfunction of insulin release as a controlling agent of blood glucose levels, so that the sugar level always increases beyond its normal condition.[20]

While treatment 1 (D1) and 2 (D2) groups showed that at the 30th minute the blood glucose level reached their peak, then the sugar level decreased gradually until the 120th minute. A gradual decrease shows the ability to absorb blood glucose levels lower than the negative and positive control groups. This indicates the potential for inhibition of alpha-amylase by all three extract combinations. Among the three dose combinations, dose 2 has the best ability to reduce blood glucose compared to the other dose combinations. Combination 2 is more effective in delaying and prolonging the time of carbohydrate digestion to reduce the rate of glucose absorption and prevent the increase in postprandial glucose levels. Figure 3 shows the differences in percentage reduction of relative blood glucose levels (% P) in the treatment groups against negative controls.

At the 30th minute, the highest percentage (11.98%) reduction of relative blood glucose level was demonstrated by dose 1. At the 60th minute, dose 2 showed the highest persentage reduction by 26.62%. At the 120th minute, dose 2 showed the highest percentage reduction by 34.80%. It was also observed that dose 2 has the highest antidiabetic activity, followed by dose 1. This effect may be attributed to the phytochemical constituents present. The phytochemicals such as flavonoids, phenolic acids, and tannins are known to regenerate the damaged *β*-cells and stopping oxidative stress on beta cells in experimental diabetic mice.[29] This decrease in blood glucose level could be attributed to some level of *in vivo* inhibition against α-amylase and α-glucosidase resulting in a suppression of hyperglycemia.[30]

The ANOVA test shows the significant value of 0.017, so it can be concluded that there is a significant difference ($p < 0.05$) between the treatments given. The Student Newman Keuls (SNK) test in Table 3 shows the best inhibitory activity was demonstrated by dose combination 2 and this combinations gave better inhibitory activity than the positive control (acarbose). This result correlated with the *in vitro* studies. It is known that in the combined extract, there are many secondary metabolites such as flavonoids, phenolic acids and tannins that can act synergistically to inhibit the activity of the alpha-amylase enzyme and prevent the development of long-term diabetes complications, including cardiovascular disease, neuropathy, nephropathy, and retinopathy.[31]

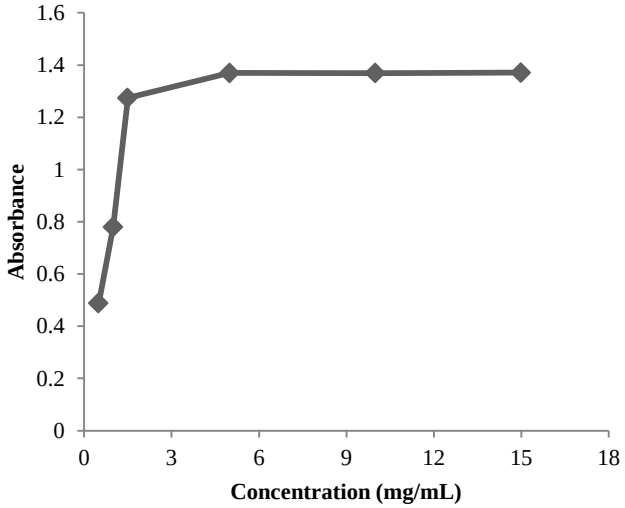

Figure 1: Optimization graph of substrate concentration with 2 U/mL alpha-amylase concentration.

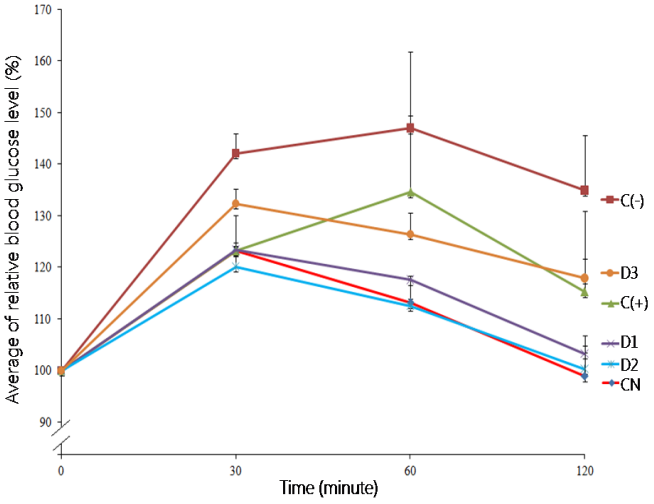

Figure 2: The Average of Relative Blood glucose Levels (%) of Diabetes Mice in Each Treatment.
Normal control (CN), Negative control (C(-)), Positive control (C(+)), Dose 1 (D1), Dose 2 (D2) and Dose 3 (D3). n = 4.

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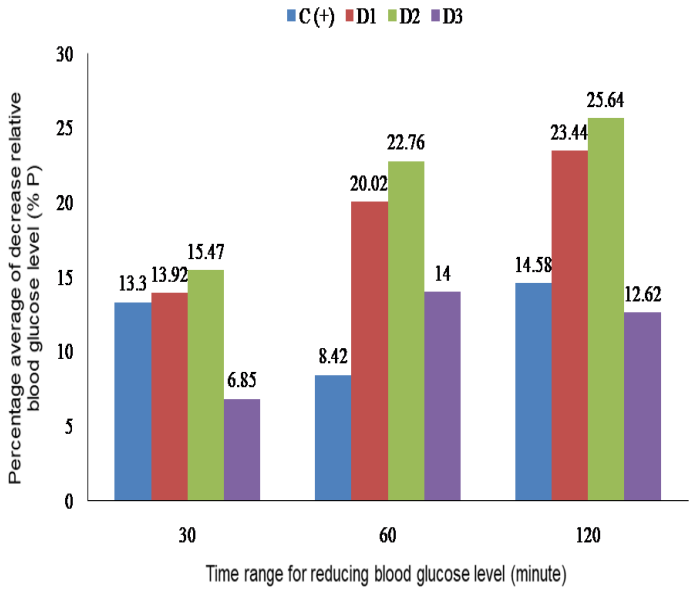

Figure 3: Graph of percentage average of decrease relative blood glucose (% P) in each treatment. Positive control (C (+)), Dose 1(D1), Dose 2 (D2), Dose 3 (D3).

Table 2: Alpha amylase inhibitory activities of Extract Combination of Morinda fruits, Fenugreek, and Black Cumin Seeds by *in vivo* studies

Conc. (µg/mL)	Dose 1	Dose 2	Dose 3	Acarbose
15.625	44.88 ± 0.97	43.87 ± 2.27	41.60 ± 0.14	31.51 ± 5.55
31.25	47.25 ± 5.12	50.81 ± 3.61	45.66 ± 0.19	35.79 ± 0.76
62.5	55.60 ± 023	54.63 ± 0.67	53.15 ± 0.09	44.36 ± 1.35
125	69.89 ± 4.90	70.58 ± 0.72	69.46 ± 4.82	61.49 ± 0.75
250	93.26 ± 3.45	93.65 ± 0.59	93.07 ± 0.30	95.75 ± 0.29
IC_{50}	38.58 ± 2.25	35.7 ± 1.33	48.78 ± 2.07	83.07 ± 2.16

Table 3: Test results of Student Newman-Keuls (SNK) relative blood glucose levels (%) for each treatment group at each observation time

Treatment	N	α = 0.005	
		1	2
D 2	4	89.4425	
D 1	4	91.2925	
D 3	4	100.1800	100.1800
C (+)	4	103.9825	103.9825
C (-)	4		109.4750
Sig.		.101	.277

Enzyme activity inhibitors are often mediated by their specificity and effectiveness, greater specificity suggests a potential for fewer side effects and low toxicity of the drugs. Natural enzyme inhibitors are involved in guiding many metabolic processes. In this study, we found that using a combination of extract would be better as an enzyme inhibitor agent. Further research on these plants should be carried out using different dosages and for different periods before recommending their use on a wide scale. This study has several limitations such as limited sample size, easy sampling techniques (non-probability). Therefore, this finding must be interpreted carefully.

Conclusion

Based on the results obtained, it could be concluded that the ethanol extract of combination of FS, MS and BCS showed alpha-amylase inhibitory activity, with dose 2 showing the best potential inhibiting activity *in vitro* and glucose lowering activity *in vivo* when compared to the other groups. However, further research is needed in order to isolate, identify, and characterize the active compounds from the extract combination.

Conflict of interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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