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Antioxidant, Antidiabetic, and Anti-obesity Potential of *Ipomoea reptans* P. Leaves

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Abstract

Ipomoea reptans Poir (local name: kangkung) is a popular leafy vegetable, a favorite to people in Asian countries. However, limited information is available on their bioactivities. In the present study, antioxidant and antidiabetic potential of *I. reptans* leaves were investigated. Different fractions (ethanol, ethyl acetate, and hexane) of *I. reptans* leaves were evaluated for their scavenging activity on DPPH radicals, whereas their reducing potential were investigated by CuPRAC, total antioxidant, and reducing power assays. The antidiabetic potential was investigated by their inhibition effect on α-glucosidase. Total phenolic and flavonoid contents of *I. reptans* leaves were solvent dependent. Ethyl acetate contained the highest phenolic content, followed by ethanol and hexane fractions. However, for flavonoid content, the order was ethanol > ethyl acetate > hexane. All fractions showed DPPH scavenging activity in a concentration dependent manner, with activities weaker than standards ascorbic acid and BHT, in the order of ethanol > ethyl acetate > hexane. All fractions showed reducing capacity. Only hexane and ethanol fractions of *I. reptans* leaves showed inhibition on α-glucosidase, with hexane showed stronger inhibition compared to acarbose. The study also found that fractions of *I. reptans* were able to inhibit lipase and trypsin, enzymes related to lipid metabolism. Findings in this study offer a prospect for *I. reptans* leaves as a functional food source for antioxidant, antidiabetic, anti-obesity purposes.

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INTRODUCTION

Free radicals are naturally generated in all living cells as a part of normal cell functions. However excessive free radicals, either from endogenous and exogenous sources, can be harmful to biological molecules such as protein, lipid, and DNA. Cellular damages such as lipid peroxidation of the membrane lipid and biomolecules have been linked to the pathogenesis and progression of various chronic and degenerative diseases, such as cardiovascular diseases and diabetes mellitus. However, the harmful effects of oxidative stress can be prevented by the consumption of antioxidant. In this case, antioxidant compounds may reduce oxidative stress conditions by stabilizing free radicals by donating protons or electrons, or by chelating pro-oxidant metal ions.

Type 2 Diabetes mellitus (T2DM) is a metabolic disorder that is characterized by high level of post prandial blood glucose. This can be due to insufficient insulin secretion, or resistance to insulin action, or a combination of both. It has been known that persistent hyperglycemia induces oxidative stress through multiple interacting pathways including activation of protein kinase C, activation of polyol pathway, and increased formation of advanced glycation end product (Mohora *et al.*, 2007). In addition, the resulting oxidative stress may further damage the pancreatic β-cells which produce insuline. In addition to oxidative stress, studies have shown that obesity could also increase the risk for T2DM (Stokes *et al.*, 2018).

Current management in T2DM and obesity include inhibition on key enzymes related to carbohydrate and lipid metabolisms. Examples of this type of medications includes acarbose and orlistat for T2DM and obesity, respectively. However, these synthetic inhibitors seem to exert major adverse side effects that potentially interfere their clinical uses such as abdominal discomfort, liver problems, and lactic acidosis (S. Saha & Verma, 2012). Consequently, there is a need for other alternatives. One possible option could come from natural inhibitors of plant origin, including vegetables and fruit. They have gained global considerations for screening bioactive compounds of medicinal attributes, including antioxidant, antidiabetic, and anti-obesity activities (Choudhury *et al.*, 2018). In addition, high consumption of vegetables and fruit has been recognized to have a positive correlation with decreased risk of chronic and nondegenerative diseases, such as cardiovascular disease, cancer, and diabetes mellitus (Carter *et al.*, 2010; van't Veer *et al.*, 2000).

Ipomoea reptans (synonym: *Ipomoea aquatica*), locally knows as 'kangkung' is a green leafy vegetable, distributed widely in South and South East Asia region, including Indonesia, Malaysia, and India. It belongs to the family Convolvulaceae. *I. reptans* is an aquatic plant, easily cultivated in a muddy or moist soil. It has a long, hollow, and tender shoot. The leaves are long heart-shaped that are rich with high nutrients, including vitamin A and C and important minerals such as calcium and iron (Rahman & Parkpain, 2004). *I. reptans* leaves is frequently consumed and is one of popular choices in Indonesian diet. However, there is limited information regarding bioactivities of *I. reptans* leaves. Previously, *I. reptans* leaves have been reported for their *in vivo* antioxidant and antihyperglycemic activities (P. Saha *et al.*, 2008)

The present study sought to investigate antioxidant activity and possible inhibition on α-glucosidase, lipase, and trypsin by *I. reptans* leaves and its fractions in several solvent systems using *in vitro* methods.

MATERIALS AND METHODS

Materials

Chemicals

All solvents and chemicals used in the experiments were of analytical grade. Folin & Ciocalteu's phenol reagent, α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside, acarbose, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and 3,5-di-tert-butyl-4- hydoxytoluene (BHT), porcine pancreatic trypsin (EC 3.4.21.4), orlistat, rutin, and sodium diclofenac were purchased from Sigma-Aldrich (St. Louis, USA). Gallic acid was obtained from Santa Cruz Biotechnology (Dallas, USA). Sodium carbonate (Na_2CO_3) was purchased from Merck (Darmstadt, Germany).

Spectrophotometer measurements were carried out using a Biochrom Libra-S22 (Cambridge, UK).

Plant material and extract preparation

The leaves of *I. reptans* were collected from Tangerang area in April 2019. Dried powdered leaves (15 g) were soaked in ethanol (400 mL) and left for 8 days with intermittent shaking. Extraction was repeated three times. The filtered supernatant was concentrated by a rotary evaporator (Rotavapor R3, Buchi Labortechnick AG, Switzerland) to obtain ethanol crude extract. The crude extract was then partitioned using hexane and ethyl acetate to obtain hexane, ethyl acetate, and ethanol fractions. Each fraction was reduced to dryness under reduced pressure, then used for the preparation of stock solution for the various analyses.

Determination of phenolic and flavonoid contents

Estimation of total phenolic content

Total phenolic content (TPC) of each fraction was determined based on a Folin-Ciocalteu method reported previously (Khatoon *et al.*, 2013). Gallic acid (12.5 – 200 µg/mL) was used to generate a standard curve. Results were presented as mg gallic acid equivalent (mgGAE)/g dried biomass.

Total flavonoid content

Total flavonoid content (TFC) of each fraction was determined based on an $AlCl_3$ colorimetric method as reported previously (Adelina Simamora *et al.*, 2018). Quercetin (3.20 – 200 µg/mL) was used to generate a standard curve. Results were presented as mg quercetin equivalent (mgQE)/g dried biomass.

Determination of antioxidant activities

DPPH radical scavenging assay

The ability of different *I. reptans* fractions to scavenge DPPH radicals was evaluated based on a reported method (Adelina Simamora *et al.*, 2018). An aliquot of each fraction solution (1 mL) was added with 3 mL of 0.6 mM DPPH solution. The reaction mixture was incubated in the dark at ambient temperature for 30 mins. The absorbance was measured at 517 nm. The absorbance of DPPH in ethanol was used a control solution and those of ascorbic acid and BHT were used as positive controls. The percentage of scavenging activity was calculated as: (A – B)/A x 100, where A is absorbance of control solution and B is absorbance of sample solution. Radical scavenging activity was presented as IC_{50} values.

Cupric ion reducing antioxidant capacity (CuPRAC) assay

CuPRAC assay was carried out based on a method described previously (Aktumsek *et al.*, 2013). A reaction mixture was prepared containing 1 mL of 10 mM $CuCl_2$, 1 mL of 7.5 mM neocuproine in ethanol, and 1 mL of 1 M NH_4OAc buffer (pH 7). Into this mixture was added extract solution (0.5 ml) and water (0.6 mL) to make a total volume of 4.1 mL. The reaction mixture was incubated at room temperature for 30 mins and the absorbance was measured at 450 nm. Trolox (10 – 320 µg/mL) was used to prepare a standard curve and results were reported as mg trolox equivalent (mgTE)/gram dried material.

Total antioxidant assay

The total antioxidant activity of each fraction was determined by a phosphomolybdenum method described previously (Prieto *et al.*, 1999). A .phosphomolybdenum reagent was prepared, containing ammonium molybdate (4 mM), sulfuric acid (0.6 M), and trisodium phosphate (28 mM). Reaction mixture was prepared in a capped tube, consisting of 3 mL of phosphomolybdenum reagent and 0.3 mL of test solution. The tube was incubated in boiling water bath

for 90 mins and after that was let to cool at room temperature. Absorbance was measured at 695 nm on a spectrophotometer. Trolox (40 – 1000 µg/mL) was used to generate a standard curve and results were expressed as mg Trolox equivalent (mgTE) / gram dried biomass.

Reducing power assay

Reducing power assay was carried out based on a ferric thiocyanate method reported previously (Gülçin *et al*., 2012). Reaction mixture was prepared containing 1 mL test solution, 2.5 mL of 0.2 M phosphate buffer pH 6.6, and 2.5 mL of 1 % (w/v) potassium ferric cyanide $K_3Fe(CN)_6$. The reaction mixture was incubated in a water bath at 50 °C for 20 mins and thereafter was cooled at room temperature. Into this mixture was added 2.5 mL of 10 % (w/v, water) tricholoracetic acid, followed by centrifugation of the mixture at 3000 rpm for 10 mins. The upper layer (2.5 mL) was taken out and mixed with 0.5 mL of 1% (w/v, water) $FeCl_3$ and 2.5 mL water. The absorbance was measured at 700 nm on a spectrophotometer. Ascorbic acid (1.56 – 100 µg/mL) was used to generate a standard curve and results were reported as mg ascorbic acid equivalent (mgAAE)/g dried biomass.

α-Glucosidase inhibition assay

Inhibition on α-glucosidase was assayed by a reported method (Simamora *et al*., 2019). In this assay, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) was used as a substrate. The reaction mixture contained 50 uL of test solution of different concentrations, 50 uL of 0.5 U/mL α-glucosidase, and 50 uL of 0.05 M phosphate buffer (pH 6.8). After pre-incubating for 5 mins at 37 °C, the mixture was added with 100 µL of 1 mM *p*NPG to start the reaction. The rection was incubated for 20 mins at 37 °C and 750 µL of 0.1 M Na_2CO_3 was added to terminate the reaction. Absorbance was measured at 405 nm on a spectrophotometer. Control solution was measured by replacing the sample with phosphate buffer. Acarbose was used as a positive control. The percentage of inhibition was calculated as: (A – B)/A x 100, where A is absorbance of control solution and B is absorbance of sample solution. Inhibition activity was presented as IC_{50} (µg/mL).

Qualitative test for lipase inhibition

Inhibition on lipase was assayed by a qualitative method of a phenol red agar plate reported previously (Gupta *et al*., 2015), with some modifications. In this method, agar (2%, w/v) was suspended with phenol red indicator (0.01%, w/v), and olive oil as a substrate (1%, v/v). Test solution was prepared by mixing in a 1:1 ration of each extract or orlistat (a synthetic lipase inhibitor) and porcine pancreatic lipase solution (200 U/ml in 0.05 M tris buffer pH 8 and NaCl 0,03 M). A 50 µL of this test solution was suspended into a circular well in the agar and the reaction was incubated for 10 mins at 37 °C. Lipolytic degradation releases fatty acids from the substrate which changes the indicator color from yellow to red.

Qualitative test for trypsin inhibition activity

Inhibition activity of *I. reptans* leaf fractions on trypsin was evaluated based on a qualitative method as reported before (Vijayaraghavan & Vincent, 2013), with some modifications. An agar plate was prepared by dissolving agar (1.5%, w/v) added with skimmed milk (5%, w/v). The agar solution was poured into petri dishes and let to solidify. Wells of 5 mm were punched. Test solution was prepared by mixing trypsin solution (10 mg in 10 ml of 100 mM tris buffer HCl pH 7.6) with fractions of *I. reptans* leaves or sodium diclofenac

(positive control) in a 1:1 ratio. A 50 µL of each test solution was loaded into each well and incubated overnight at 37 °C. Trypsin inhibition was observed by a decrease in zone diameter in the presence of inhibitors.

Statistical analysis

All experiments were conducted in three replicates and results were presented as mean ± SD. The significance of difference among multiple averages was determined by analysis of variance (ANOVA), followed by a Tukey post hoc test at 5% significance level.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

The total phenolic and flavonoid contents of *I. reptans* leaves with respect to solvents used for fractionation are presented in Table 1. It is clear that solvents significantly affected TPC and TFC obtained ($p < 0.05$). Among three different solvents, ethyl acetate appeared to be the best solvent to extract phenolic compounds (Table 1), followed by ethanol and hexane. However, in TFC results, maximum TFC was obtained in ethanol followed by ethyl acetate and hexane. It was reported previously that the use of polar solvent such as methanol and water resulted in high phenolic content but low flavonoid content (Dasgupta & De, 2007; Prasad *et al.*, 2005). In the presence study, less polar solvent such as ethyl acetate was more effective in extracting phenolics, whereas ethanol was more effective for flavonoids. This could be due to the difference in method used for extracting the phenolic and flavonoid compounds.

Put Table 1 somewhere here……

***In vitro* antioxidant activities**

One of antioxidant mechanism of actions is by removing free radicals, which can be achieved by transferring protons or electrons from antioxidant compounds to the free radicals. In the present study, radical scavenging activity of *I. reptans* fractions was evaluated using stable DPPH radicals. The use of DPPH radicals may be relevant to represent a lipophilic radical initiated by lipid auto oxidation (Shukla *et al.*, 2016). It was proposed that scavenging mechanism for DPPH to form the non-radical DPPH-H is predominantly through proton transfer (Marxen *et al.*, 2007).

The scavenging capacity of *I. reptans* leaf fractions on DPPH radicals was presented as % inhibition and IC_{50} values (Table 2). It is clear all fractions exhibited inhibition on DPPH radicals in a concentration dependent manner. This is indicative of proton donating capacity of all fractions. Based on their IC_{50} values, it is worth noting that DPPH radical scavenging activities of ethanol and hexane samples did not differ significantly ($p > 0.05$). The order as seen in the table is ethanol > hexane > ethyl acetate.

Phenolic and flavonoid compounds are known to be strong proton donor, (Paixão *et al.*, 2007). In fact, quercetin derivative isolated from *I. reptans* was shown to have a potent DPPH radical scavenging activity (Prasad *et al.*, 2005). Results in the present study suggest that antioxidant activity may not solely attributed to phenolic and flavonoid compounds and that other compounds may also contribute to the scavenging activity. Previous study has reported that non-phenolic compounds isolated from plants had antioxidant activity (El-Sayed *et al.*, 2008).

Put Table 2 somewhere here……

In addition to radical scavenging activity, antioxidant activity of *I. reptans* extracts were also evaluated by their reducing capacity. Compounds having reducing capacity indicate their ability to act as an electron donor to any oxidized intermediates to form more stable species of lower oxidation states. In the present study, CuPRAC, phosphomolybdenum, and ferric thiocyanate methods were employed to investigate the reducing potential of ethanol, ethyl acetate, and hexane fractions of *I. reptans*. These methods are different in term of reaction conditions and the metal ions used. However, in all methods, higher values indicate a stronger reducing capacity.

Results in Table 3 suggested that *I. reptans* had reducing capacities. This indicates that antioxidant compounds in all fractions are able to transfer electron to metals of higher oxidation number, and reduce them to lower oxidation states. In this case, Cu(II) to Cu(I), Mo(VI) to Mo(V), and Fe(III) to Fe(II), for CuPRAC, Total antioxidant, and Ferric thiocyanate activities, respectively. Results also indicated that solvent had pronounced effect on the reducing capacity, as indicated by significantly different activities observed among extracts. Based on CuPRAC assay, hexane fraction exhibited the highest activity, followed by ethanol and ethyl acetate fractions. The same order was observed based on reducing power assay. However, a slight difference was observed using the phosphomolydenum assay, where the order follows ethanol > hexane and ethyl acetate fractions. The difference could be due to the sensitivity of each metal ion used in the respective method (Choirunnisa *et al.*, 2016). It is known that the ease of each metal ions to be reduced to lower oxidation state depend on redox potential of each metal ion.

Previous study reported antioxidant activity of methanol extract of *I. reptans* leaves using an animal model (P. Saha *et al.*, 2008). This study used STZ induced diabetic rats and observed a decrease in MDA level and an increase in GSH level in the liver, pancreas, and kidney tissue of extract treated rats, indicating a lower oxidative stress condition due to extract treatment. These results complement with those observed in DPPH scavenging activity, thus *I. reptans* can act as radical scavengers and reducing agents.

Put Table 3 somewhere here……

***In vitro* antidiabetic activity**

As α-glucosidase hydrolyzes the catalytic degradation of polysaccharide or oligosaccharide into glucose, this enzyme has become a therapeutic target for regulating blood glucose level. *In vitro* antidiabetic activity of ethanol, ethyl acetate, and ethanol fractions of *I. reptans* was evaluated by examining their inhibition effect on α-glucosidase activity. In this study, acarbose, a standard α-glucosidase inhibitor was used as a positive control.

Results in Table 4 shows that only hexane and ethanol fractions exhibited inhibition activity on α-glucosidase. In both cases, % of inhibition activities increased with increasing extract concentrations. However, hexane showed a significantly stronger activity when compared to ethyl acetate and acarbose ($p > 0.05$). On the other hand, ethyl acetate showed no inhibition activity on α-glucosidase.

This finding was supported by previous study reporting antihyperglycemic activity of methanol extract of *I. reptans* leaves using STZ induced diabetic rats (P. Saha *et al.*, 2008). It is possible that the observed hypoglycemic activity in this animal model may be due to inhibition activity of *I. reptans* leaves on α-glucosidase. Various phenolic and flavonoid compounds have been reported to be able to inhibit α-glucosidase *in vitro* (Limanto *et al.*,

2019; Yin *et al*., 2014). However, lack of activity observed in ethyl acetate indicates that α-glucosidase inhibition activity is not only attributed to phenolic and flavonoid compounds.

Put Table 4 somewhere here……

***In vitro* anti-lipase activity**

In vitro anti-obesity activity for ethanol and ethyl acetate fractions were conducted based on inhibition activity on lipase. In the present study, a qualitative method using a phenol red agar plate was used and the results can be seen in Figure 1. Figure 1a shows an olive oil – lipase system in the absence of inhibitor. No inhibition was observed by strong intensity in the red halo and large halo diameter. However, in the presence of inhibitors, orlistat (well B), ethyl acetate (well C) and ethanol fractions (well D), positive inhibitions on lipase were observed. In each case, a decrease in halo diameter and less intense red halo was observed. Inhibition on lipase decreased lipolytic degradation of olive oil, thus generating fewer fatty acids. Previously, many plants have been studied for their anti-lipase activity (Rajan *et al*., 2020). However, to date, no studies yet reported on the anti-lipase activity of *I. reptans*. Finding in the present study may serve as a preliminary screening for further investigations on the anti-obesity activity of *I. reptans* leaves.

Put Figure 1 somewhere here……

In vitro anti-trypsin activity

Trypsin has been studied for its role in the treatment for obesity (Cristina Oliveira de Lima *et al*., 2019). Previous result in an *in vivo* study suggested that treatment with synthetic trypsin inhibitor was able to reduce intake and meal size of obese rats, possibly by modulating satiety hormone such as cholecystokinin (McLaughlin *et al*., 1983). In the present study, fractions of *I. reptans* were evaluated for their potential trypsin inhibition activity and results were presented in Figure 2. Figure 2a shows trypsin system in the absence of inhibitor, exhibited by a maximum zone diameter. However, in the presence of inhibitors, decreases in zone diameter were clearly observed, as can be seen for wells B, C, and D, indicating inhibition on trypsin activity. To date, very few studies reported on the anti-trypsin activity of plant extracts (Miedzianka *et al*., 2020). The positive results obtained in this study can be a starting point to further study trypsin inhibition effect of *I. reptans*, in particular in conjunction with anti-obesity activity.

Put Figure 2 somewhere here……

CONCLUSION

The present study proved the antioxidant and antidiabetic properties of *I. reptans* leaves. Study revealed that solvents used in the partition had a significant influence on the antioxidant and α-glucosidase inhibition activity. The study can conclude that *I. reptans* leaves could be a natural resource for antioxidant, antidiabetic, and antiobesity agent.

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