



# Enzyme inhibitory, antioxidant, and antibacterial activities of ethanol fruit extract of *Muntingia calabura* Linn

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## ARTICLE INFO

**Article Type:**  
Original Article

**Article History:**  
Received: 27 July 2019  
Accepted: 10 October 2020

**Keywords:**  
DNA protection  
Fruit  
 $\alpha$ -Glucosidase  
Kersen  
Lipase  
Xanthine oxidase

## ABSTRACT

**Introduction:** *Muntingia calabura* is used for its many medicinal advantages. So far, limited studies have been done for the bioactivities of *M. calabura* fruit. This study aimed to investigate the enzyme inhibitory, antioxidant, and antibacterial activities of *M. calabura* fruit.

**Methods:** Ethanol extract of *M. calabura* fruit was tested for its inhibitory enzyme activities against key enzymes linked to human pathologies, such as diabetes ( $\alpha$ -glucosidase and  $\alpha$ -amylase), hyperuricemia (xanthine oxidase), and obesity (lipase). The antioxidant properties were investigated using different *in vitro* assays, i.e. 1,1-diphenyl-2-picrylhydrazyl (DPPH), cupric ion reducing antioxidant capacity (CUPRAC), reducing power, phosphomolybdenum, metal chelating and DNA-damage protection assays. The fruit was also evaluated for its antibacterial activities against several gram positive and negative bacteria.

**Results:** The total phenolic and flavonoid contents of the extract were 10.85 mg gallic acid equivalent (GAE)/g and 3.30 mg quercetin equivalent (QE)/g, respectively. The fruit extract showed good inhibition against  $\alpha$ -glucosidase and  $\alpha$ -amylase ( $IC_{50}$  16.74 and 46.49  $\mu$ g/mL, respectively), with activities stronger than acarbose (100.38 and 152.46  $\mu$ g/mL, respectively). It exhibited weak inhibitory activity against xanthine oxidase ( $IC_{50}$  0.91 mg/mL) and lipase ( $IC_{50}$  16.48 mg/mL), weaker than the references used for respective test ( $IC_{50}$  allopurinol 5.31  $\mu$ g/mL and orlistat 0.17  $\mu$ g/mL). The extract showed antibacterial activities against *Chromobacterium violaceum*, *Staphylococcus aureus*, *Streptococcus mutans*, *Staphylococcus epidermidis* and *Escherichia coli*. The ethanol extract showed weaker antioxidant activity, when compared to ascorbic acid and butylated hydroxytoluene (BHT). However, the extract was able to protect DNA-damage.

**Conclusion:** The study concludes that *M. calabura* fruit exhibits antioxidant, antibacterial, and enzyme inhibitory properties, thus can be a good source for pharmacological uses.

### Implication for health policy/practice/research/medical education:

The bioactivities (enzyme inhibitory, antioxidant, and antibacterial activities) of *M. calabura* fruit proved in this study support the ethnopharmacological use of this fruit, therefore it is recommended for further exploration as a natural source for food supplement in pharmaceutical industry.

*Please cite this paper as:* Simamora A, Santoso AW, Rahayu I, Timotius KH. Enzyme inhibitory, antioxidant, and antibacterial activities of ethanol fruit extract of *Muntingia calabura* Linn. J Herbmed Pharmacol. 2020;9(4):346-354. doi: 10.34172/jhp.2020.44.

## Introduction

Plants have long been explored as valuable sources for pharmacological active compounds (enzyme inhibitors, antioxidant, antibacterial agents). De Luca et al reported that 25% of the commercial drugs are derived from natural products (1), and many of them have been used for treatment of chronic degenerative diseases and infectious diseases. A lot of these pathologies are linked

to oxidative stress which is an important factor in cell damage and has been implicated in the development of degenerative diseases, such as diabetes mellitus, gout, obesity, atherosclerosis, and cancer (2,3). Synthetic compounds have been produced for the management of these diseases. However, they may have some side effects and toxic properties to human. Furthermore, multi-drug bacterial resistance requires new antibacterial agents.

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Plants can be potential sources for the quest of therapeutic agents that could be safer than the synthetic sources (4).

Enzyme inhibition is a central therapeutic strategy for the treatment of chronic degenerative diseases. Digestive enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase are the key enzymes in the hydrolysis of starch and oligosaccharide into glucose. Inhibition of these enzymes reduces the postprandial blood glucose, due to reduction in absorption of glucose. Xanthine oxidase is responsible for the catalytic formation of uric acid. Inhibition of uric acid formation through xanthine oxidase pathway can be a crucial strategy in overcoming hyperuricaemia and gouty arthritis. In addition, inhibition of lipase, which is responsible for the catalytic degradation of triglycerides, has been an important approach for the treatment of obesity and hyperlipidaemia. Recently, there is a growing interest in plant-derived inhibitors to modulate physiological effect of enzymes linked to these diseases. Natural inhibitors such as those from medicinal plants can be viable alternative agents as they produce relatively fewer adverse effects and are more economical agents compared to synthetic inhibitors.

*Muntingia calabura* (family Elaeocarpaceae) is distributed throughout tropical areas, such as in western part of South America, the Caribbean and Southeast Asia regions. *M. calabura* is a fast-growing tree, that can reach up to 12 m high. Its branches are horizontally spreading make them ideal for a shade tree function. The fruit of *M. calabura* (local name: kersen or talok) is edible due to its sweet taste. Ethnobotanical studies have reported the use of *M. calabura* plant for the treatment of a number of diseases. The leaves, flowers, roots, and barks are used to treat insipient cold, headache, liver diseases and ulcer (5). Different parts of *M. calabura* have been scientifically demonstrated to exert various pharmacological properties. Extracts of the leaves have anti-nociceptive (6), anti-carcinogenic (7), anti-bacteria (8), antioxidant and anti-inflammatory activities (9). The root of *M. calabura* has also been shown to have flavonoids with anticancer activity (10). Despite the numerous pharmacological studies on the leaves, less has been known about the functional role of *M. calabura* fruit, with only several studies reported on antioxidant (11) and anti-inflammatory activities (12). The present study aimed to investigate the bioactivity of the fruit extract of *M. calabura*, in particular its potential enzyme ( $\alpha$ -glucosidase,  $\alpha$ -amylase, xanthine oxidase, and lipase) inhibitory and antibacterial activities. Further, its antioxidant activity was also evaluated using different *in vitro* assays, including its protective effect against DNA damage. This study may contribute to the development of new resources for dietary health supplement for the prevention or adjunctive therapy of chronic diseases such as diabetes and gout which are associated with oxidative stress and infection.

## Material and Methods

### Chemicals

Acarbose,  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ , allopurinol,  $\alpha$ -amylase from porcine pancreas (EC 3.2.1.1), butylated hydroxytoluene (BHT), pBR322 plasmid DNA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), EDTA, Folin-Ciocalteu reagent, lipase from porcine pancreas (EC 3.1.1.3), *p*-nitrophenyl  $\alpha$ -D-glucopyranoside (*p*-NPG), *p*-nitrophenyl palmitate (*p*-NPP), orlistat, quercetin, xanthine, and xanthine oxidase from bovine milk (EC 1.17.3.2) were purchased from Sigma-Aldrich (St Louis, USA). Ammonium molybdate,  $\text{NaNO}_2$ , and starch were purchased from Merck company (Darmstadt, Germany). Gallic acid was purchased from Santa Cruz Biotechnology. All remaining reagents were of the highest purity available (>98%). All solvents were of analytical grade. Spectrophotometer measurements were carried out using a Biochrom Libra-S22 (Cambridge, UK).

### Extract preparation

The fruit of *M. calabura* was obtained from the Botanical Garden of Jember University, Jember, East Java, Indonesia in August 2018. Fresh fruits were dried at 40°C using a food dehydrator. They were pulverised and then macerated with ethanol for 72 hours. After filtering through a filter paper (Whatman no 1), the solvent was evaporated under reduced pressure. The extract was then kept in the refrigerator at 4°C prior to analysis.

### Estimation of the total phenolic and flavonoid contents

The total phenolic content (TPC) of *M. calabura* fruit extract was estimated by a modified Folin-Ciocalteu method as described in the previous report (13). Gallic acid was used as a reference compound. The TPC was estimated from an equation derived from a gallic acid standard curve ( $R^2 = 0.9976$ ). Data were presented as mg gallic acid equivalent (mgGAE)/g of extract yield.

The total flavonoid content was determined based on an aluminium chloride colorimetric method reported previously (14). Quercetin was used to construct a standard curve ( $R^2 = 0.9998$ ) and used for the estimation of total flavonoid content of *M. calabura* fruit extract. TFC of the extract was presented as mg quercetin equivalent (mgQE)/g of yield.

### Enzyme inhibitory activity assays

#### $\alpha$ -Glucosidase inhibitory activity assay

The inhibition of the ethanol extract of *M. calabura* fruit on  $\alpha$ -glucosidase (from *Saccharomyces cerevisiae*) was determined based on a modified method described in a previous report (15). Activity was expressed as  $\text{IC}_{50}$  and the value was compared to that of acarbose, a synthetic inhibitor.

#### $\alpha$ -Amylase inhibitory activity assay

The method for measuring  $\alpha$ -amylase inhibitory activity

of *M. calabura* fruit extract was adapted from a previous report (16), using porcine pancreatic  $\alpha$ -amylase and starch as a substrate. Acarbose was tested as a positive control for comparison, based on  $IC_{50}$  values.

#### *Xanthine oxidase inhibitory activity assay*

The inhibitory activity of the ethanol extract of *M. calabura* fruit on bovine milk xanthine oxidase was determined based on a method as described before (17). A solution of allopurinol, a known synthetic xanthine oxidase inhibitor, was used as a positive control.

#### *Lipase inhibitory activity assay*

The lipase inhibitory activity of *M. calabura* fruit extract was determined based on a reported method (18) with some modifications as follows. The reaction used *p*-nitrophenyl palmitate (*p*NPP) as a synthetic substrate. Extract of different concentrations in dimethyl sulfoxide (DMSO; 100  $\mu$ L) was mixed with 300  $\mu$ L porcine pancreatic lipase (200 U/mL in tris-buffer, 50 mM pH 8, ionic strength NaCl 30 mM) and the mixture was kept for 5 mins at 37°C. To start the reaction, 200  $\mu$ L of *p*NPP (10 mM in DMSO) was added, and the mixture was incubated for 20 mins at 37°C. Ethanol (500  $\mu$ L) was added to terminate the reaction, and the absorbance was measured at 405 nm on a spectrophotometer. Results were obtained from triplicate testings. Orlistat, a well-known lipase inhibitor was used as a positive control. The percentage of inhibition, plotted against concentration and  $IC_{50}$ , was calculated from the linear regression equation.

#### *Phenol red olive oil plate assay*

In this method, pancreatic lipase activity was assessed using olive oil as a substrate as described previously (19) with minor modification. The formation of fatty acids as a result of hydrolysis of olive oil by lipase changes the colour of a pH indicator dye. In brief, agar plate (2%) was prepared using olive oil (1%) and phenol red as an indicator (0.01%). The extract was mixed with pancreatic lipase (200 U/mL in 50 mM tris buffer pH 8 and NaCl 30 mM) in a 1:1 ratio. The plate was incubated for 10 mins at 37°C. Degradation of substrate was indicated by the change in colour of the indicator from yellow to red. Lipase inhibitory activity was observed by the decrease in the halo formation in the presence of the lipase inhibitor.

#### *Antioxidant activity assays*

The radical scavenging activity of the *M. calabura* fruit extract was determined based on a DPPH assay. Ascorbic acid and BHT were used as standards. Scavenging activity was expressed as  $IC_{50}$  value. The reducing capacity of the *M. calabura* fruit extract was determined based on several reported methods, i.e., CUPRAC method (20) and total antioxidant capacity/phosphomolybdenum assay, where mg trolox equivalent (mgTE) /g extract yield was

used as a unit of measurement. In addition, reduction capacity was also tested via Fe(III)-Fe(II) transformation (ferric thiocyanate assay), expressed as mg ascorbic acid equivalent (mgAAE)/g extract yield. Ferrous ion chelating activity of ethanol extract of *M. calabura* fruit was determined as described in the previously reported study. Result was expressed as  $IC_{50}$  value and compared with the standard EDTA. All antioxidant activity procedures except for CUPRAC assay were explained in a previous paper (13).

#### *DNA damage protective effect assay*

The ability of the ethanol extract of *M. calabura* fruit to protect the supercoiled pBR322 plasmid DNA against  $H_2O_2$  was estimated based on the DNA nicking assay as described before (21) with minor modifications. The reaction mixtures (15  $\mu$ L) contained 5  $\mu$ L of phosphate buffer saline (PBS, 10 mM, pH 7.4), 1  $\mu$ L of plasmid DNA (0.5  $\mu$ g), 5  $\mu$ L of extract of different concentrations, 2  $\mu$ L of  $FeSO_4$  (1 mM), and 2  $\mu$ L of  $H_2O_2$  (1 mM). The reaction mixture was incubated at 37°C for 30 minutes. At the end of incubation, 2  $\mu$ L of a loading buffer containing glycerol (50%, v/v), EDTA (40 mM), and bromophenol blue (0.05%) was added to stop the reaction. The reaction mixtures were electrophoresed for 60 minutes (60 V) on 1% agarose gel containing 0.5  $\mu$ g/mL ethidium bromide in Tris/acetate/ EDTA gel buffer, using an electrophoresis instrument (DYCP-31A, Beijing Liuyi, China). The DNA in the gel was visualized and photographed under ultraviolet light.

#### *Antibacterial assays*

##### *MIC and MBC determinations*

The antibacterial activity of the ethanol extract of *M. calabura* fruit was determined by evaluating its minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC). The antibacterial activity was studied against six bacterial strains, i.e., *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus mutans* ATCC 14721, *Chromobacterium violaceum* and ATCC 2472.

The MIC analysis was performed on the selected bacteria based on a dilution broth method using a U-bottom 96-well plate. The initial solution of the extract was prepared in ethanol, then 50  $\mu$ L of the extract was pipetted into the first column of the 96-well plate. To all other wells, a volume of 50  $\mu$ L of Mueller-Hinton broth (Himedia M391) was added. Inoculum at the maximum 24 hours old was used to prepare bacterial suspension at 0.5 McFarland using a BioSan Den-1B. Suspension was added with 1:20 of water: Tween 80 and 10  $\mu$ L of the suspension was suspended into each well. Serial dilutions were then performed for each well, taking each 50  $\mu$ L of the sample which was diluted serially in a descending

concentration to give different concentrations of the extract. The mixture was incubated for 24 hours at 37°C. A negative (growth) control was prepared containing inoculum without antibacterial solution. In addition, solvent toxicity (ethanol without extract) against bacteria was also evaluated to ensure no antibacterial effect of the solvent. MIC was determined as the lowest extract concentration showing no apparent growth (turbidity) after 24 hours incubation. MBC was determined as the lowest concentration showing no observable growth on the agar subculture.

### Statistical analysis

All experiments were run in triplicate. Results were expressed as mean  $\pm$  SD. Regression linear method was used to calculate  $IC_{50}$  values and to generate standard curves.

## Results

### Total phenolic and flavonoid compounds

The yield of *M. calabura* fruit macerated in ethanol was 21.15% (w/w). The colour of the extract was yellowish brown. The extract was evaluated for its total phenolic and flavonoid contents, obtaining  $10.85 \pm 0.02$  mgGAE/g extract and  $3.30 \pm 0.03$  mgQE/g extract, respectively.

### Enzyme inhibitory activity

The present study investigated the antidiabetic activity of *M. calabura* fruit extract by evaluating its inhibitory activity against  $\alpha$ -glucosidase and  $\alpha$ -amylase. As can be seen in Table 1, the extract exhibited strong activity, obtaining a much lower  $IC_{50}$  value compared to acarbose. Acarbose is a common  $\alpha$ -glucosidase inhibitor and used as a positive control. In the same way, ethanol extract of *M. calabura* fruit showed a strong  $\alpha$ -amylase inhibition, more potent than acarbose.

The extract was also tested for its inhibitory activity on xanthine oxidase. The fruit extract was able to inhibit xanthine oxidase, although the activity was much weaker than the positive control allopurinol.

Evaluation of lipase inhibitory activity on pancreatic lipase showed that the extract was able to inhibit lipase in a concentration dependent manner. Incremental increases of extract concentration gave rise to an increase in percentage of inhibition (Figure 1A). However, the

activity was very weak as indicated by its  $IC_{50}$  value when compared to orlistat, a positive control for this test. Lipase inhibitory activity of the extract was further confirmed using the phenol red agar assay (Figure 1B). It can be seen that in the presence of *M. calabura* fruit extract, the red halo was decreased, indicating an inhibitory activity by the extract, as similarly observed in the presence of orlistat.

### Antioxidant activity

Due to various mechanisms of antioxidant actions, antioxidant capacity of a sample cannot be evaluated by a single method. Therefore, different assays were employed to assess the antioxidant capacity of the ethanol extract of the *M. calabura* fruit i.e., DPPH radical scavenging, CUPRAC, ferric thiocyanate, phosphomolybdenum and ferrous ion chelating assays (Table 2).

One of the pathways of antioxidant mechanism of action is through scavenging free radicals. The DPPH assay measures the ability of the antioxidant compounds in the extract to scavenge free radicals by donating proton to DPPH radicals, thus quenching the radicals into more stable form. When compared with the positive control, BHT and ascorbic acid, the DPPH  $IC_{50}$  value of the extract was higher, indicating lower activity than the controls.

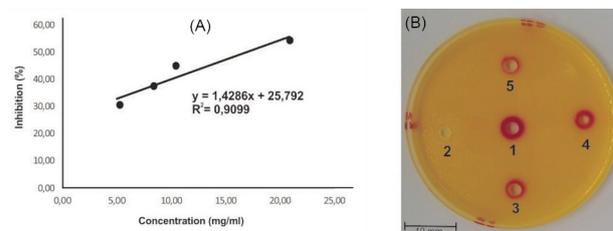
The reductive ability is an important indicator of antioxidant potential. In this study, CUPRAC, ferric thiocyanate, and phosphomolybdenum assays were used to measure the reductive capacity of the *M. calabura* extract. The presence of reductants in the extract causes the reduction of metal ions into their reduced form by donating an electron. In this case, the metal ions reduction involved in the reactions were Cu(II) to Cu(I), Fe(III) to Fe(II), and Mo(VI) to Mo(V) in CUPRAC, ferric thiocyanate, and phosphomolybdenum assays, respectively. As can be seen in Table 2, the *M. calabura* fruit extract showed capacity to alter these transition metals into their reduced states using all the assays.

Transition metal ions can act as a catalysator in lipid peroxidation reaction, thus chelating agents for these metal ions play role as secondary antioxidants. Plant extracts contain compounds such as polyphenols that are capable of chelating metal ions. As can be seen from the

**Table 1.** Enzyme inhibition by ethanol extract of *M. calabura* fruit

Enzymes	Unit	$IC_{50}$	Positive control
$\alpha$ -Amylase	$\mu\text{g/mL}$	$46.49 \pm 0.09$	Acarbose $152.46 \pm 8.43$
$\alpha$ -Glucosidase	$\mu\text{g/mL}$	$16.74 \pm .49$	Acarbose $100.38 \pm 2.19$
Xanthine oxidase	$\text{mg/mL}$	$0.91 \pm 0.04$	Allopurinol $5.31 \pm 0.49$ ( $\mu\text{g/mL}$ )
Lipase	$\text{mg/mL}$	$16.48 \pm 0.17$	Orlistat $0.17 \pm 0.65$ ( $\mu\text{g/mL}$ )

Reported values are expressed as means  $\pm$  SD (n=3 replicates).



**Figure 1.** Anti-lipase activity of *M. calabura* fruit extract as assayed by (A) in vitro pancreatic lipase inhibitory activity. (B) phenol red oil plate assay, 1: no inhibitor, 2: extract without lipase substrate, 3: orlistat, 4: extract (20.92 mg/mL), 5: extract (10.46 mg/mL). Results are from triplicates experiments.

**Table 2.** Antioxidant activity of ethanol extract of *M. calabura* fruit

Antioxidant assay	Unit	Result
DPPH assay	IC <sub>50</sub> µg/mL	367.36 ± 0.14
Ascorbic acid	IC <sub>50</sub> µg/mL	53.24 ± 0.82
BHT	IC <sub>50</sub> µg/mL	21.36 ± 0.80
CUPRAC assay	mgTE/g	3.76 ± 0.02
Ferric reducing capacity	mgAAE/g	8.56 ± 0.17
Phosphor molybdenum assay	mgAAE/g	6.79 ± 0.07
FIC assay	IC <sub>50</sub> mg/mL	3.84 ± 0.19
EDTA	IC <sub>50</sub> mg/mL	0.064 ± 0.00

Abbreviations: BHT, Butylated hydroxytoluene; CUPRAC, Cupric ion reducing antioxidant capacity; FIC, Ferrous ion chelating activity; EDTA, Ethylenediaminetetraacetic acid.

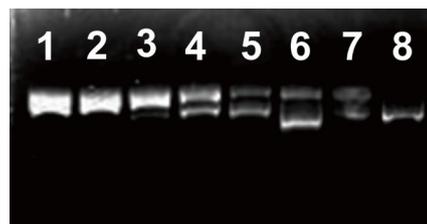
Reported values are expressed as means ± SD (n=3 replicates).

result, the Fe(II) chelating activity of *M. calabura* fruit extract was not as good as EDTA (positive control), *i.e.* its IC<sub>50</sub> value was higher than that of EDTA. However, decrease in the colour formation in the presence of the extract indicates the iron binding capacity of the extract.

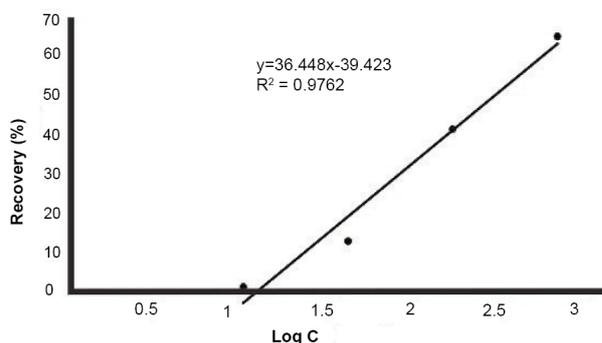
The ability of *M. calabura* fruit extract to protect DNA from damage induced by •OH was evaluated by an *in vitro* DNA damage assay using pBR322 plasmid DNA. Exposure of plasmid DNA to •OH radicals leads to oxidatively induced break in DNA strands, from the supercoiled into open circular or relaxed form. Figure 2 depicts the electrophoretic pattern of plasmid DNA, in the presence and absence of the fruit extract. Band in lane 7 shows DNA derived from pBR322 plasmid in the absence of Fe(II) and H<sub>2</sub>O<sub>2</sub> (control), which is mainly in the supercoiled form. The presence of the Fenton reagent resulted in the cleavage of the supercoiled DNA into a more relaxed form (lane 1), indicating that •OH radicals generated by means of Fenton reaction causes DNA cleavage. Lane 2 to 6 shows plasmid DNA treated with extract in increasing concentrations. The addition of fruit extract recovered DNA from damage, as can be seen from the retention of the supercoiled form band (Lane 6). The protective effect of the extract was concentration-dependent as exhibited in Figure 3. The percentage of plasmid DNA recovery increased with the increase in fruit extract concentration (in Log C).

#### Antibacterial activity

The extract of *M. calabura* fruit was investigated for its antibacterial activities against gram positive (*S. aureus*, *S. epidermidis*, and *S. mutans*) and gram negative (*C. violaceum* and *E. coli*) bacteria, which were selected based on their relevance to human pathogens. As seen on Table 3, the fruit extract exhibited considerable antibacterial activity against the tested bacteria. The experiments revealed that *C. violaceum* was the most sensitive as can be seen from the lowest MIC and MBC values. Whereas, the lowest activity was observed against *S. epidermidis* and *E. coli*.



**Figure 2.** DNA damage protective effect of ethanol extract of *M. calabura* fruit. Lane 1: plasmid pBR322 treated with Fenton reagent; Lane 2-6: plasmid pBR322 treated with Fenton reagent and extract in increasing concentrations. Lane 7: the native plasmid pBR3G322. Concentrations of the extract (lane 2 to 6) from 0,01; 0,04; 0,16; 0,65 and 10,46 mg/mL, respectively.



**Figure 3.** Recovery percentage of plasmid pBR322 in the presence of *M. calabura* fruit extract in increasing concentration (log C = 1.00 to 4.02).

#### Discussion

The bioactivities of the plants, such as their enzyme inhibitory and antioxidant activities are mainly contributed by the phytochemicals present in them, such as those of phenolic and flavonoid derivatives. It has been reported that antioxidant capacity has a positive correlation with the content of phenolics in the plants (22). Moreover, a body of literatures have reported antidiabetic activity of polyphenolics in plants (23). The present study found significant amount of phenolics and flavonoids in the ethanol extract of *M. calabura* fruit. However, the total phenolic content of the ethanol extract of *M. calabura* fruit was lower than those found in the leaf and stem extracts (8).

Diabetes mellitus is a metabolic syndrome typified by chronic hyperglycaemia, which is caused by deficiency in insulin secretion and/or insensitivity in insulin action (type II DM). This illness has become a major health problem with worldwide prevalence. One of therapeutic approaches in controlling blood glucose level is by inhibition of key enzymes involved in the hydrolysis of polysaccharides, in particular  $\alpha$ -glucosidase and  $\alpha$ -amylase. However, synthetic inhibitors (such as acarbose, viglibose, etc) were reported to cause gastrointestinal complications such as flatulence, diarrhoea, and abdominal cramping. Therefore, there is a need for finding alternative inhibitors

**Table 3.** Antibacterial activities (MIC and MBC values) of ethanol extract of *M. calabura* fruit

Bacterial strain	Ethanol Extract of <i>Muntingia calabura</i>		Ciprofloxacin	
	MIC (mg/mL)	MBC (mg/mL)	MIC ( $\mu$ g/mL)	MBC ( $\mu$ g/mL)
<i>Chromobacterium violaceum</i> ATCC 12472	0.023	0.207	0.05	0.01
<i>Staphylococcus aureus</i> ATCC 25923	0.069	0.207	0.00039	0.00078
<i>Streptococcus mutans</i> ATCC 14721	0.069	0.207	0.003125	0.00625
<i>Staphylococcus epidermidis</i> ATCC 12228	0.622	1.866	0.025	0.050
<i>Escherichia coli</i> ATCC 25922	0.622	1.866	0.001563	0.003125

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration

with less toxic effects. In response to this, it is important to evaluate the antidiabetic potential of medicinal plants. The present study found that *M. calabura* fruit exhibited strong inhibitory activity against  $\alpha$ -glucosidase and  $\alpha$ -amylase, stronger than acarbose, a prescribed  $\alpha$ -glucosidase inhibitor.

A previous study reported hypoglycaemic effect of extracts, prepared from the leaves of *M. calabura* using animal models (24). No studies have previously reported yet the anti-diabetic effect of the *M. calabura* fruit. Therefore, the present study is complementary to the previous findings. Our results proved that ethanol extract of *M. calabura* fruit might be considered as a good source for  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitor.

Xanthine oxidase is an enzyme responsible for the catalytic conversion of hypoxanthine and xanthine to uric acid. Elevated uric acid in blood leads to hyperuricemia and gout disease development. In oxidizing xanthine, superoxide ( $O_2^-$ ) and hydrogen peroxide are generated, leading to an increased cellular oxidative stress. This condition contributes to the progression of diabetes complications. Evidence has been reported for the causal relationship between type 2 diabetes and hyperuricemia (25). Allopurinol is the first line urate-lowering drug. However, the use of this synthetic xanthine oxidase inhibitor was reported to cause adverse effects, such as abnormal liver function and severe cutaneous reactions (26). Therefore, there is an interest in the development of other new xanthine oxidase inhibitors. The present study revealed that the fruit extract of *M. calabura* possessed inhibition potential against xanthine oxidase.

Obesity is now a global health problem. Obesity is one of the risk factors for metabolic syndrome and is associated with several chronic conditions such as type II diabetes mellitus, hyperlipidaemia, hypertension, and coronary artery diseases. Hyperlipidaemia has been reported to induce oxidative stress which contributes to the diabetic vascular complications. In hyperlipidaemia, reactive oxygen species (ROS) are generated by a non-enzymatic mechanism via advanced glycation end product (AGE) pathway (27). One strategy to treat obesity is by suppressing the digestion of triglycerides into glycerol and fatty acids, thus reducing its absorption in the small intestine. This can be achieved by inhibiting

pancreatic lipase which is responsible for the degradation of almost 70% of dietary triglycerides. Orlistat, the only long-term anti-obesity drug approved by European Medicines Agency (EMA) and the US FDA, acts through the inhibition of pancreatic lipase. The use of this drug, though has been shown to be effective, is compromised by its adverse effects such as abdominal pain, oily stools, bloating, flatulence, and decrease in absorption of fat-soluble vitamins (28). In this direction, there is a growing interest in the development of lipase inhibitors with better properties, in particular from natural resources. In the present study, two assays were employed for the evaluation of *M. calabura* activity on inhibiting lipase activity. It is apparent that the fruit exhibited inhibitory potential against pancreatic lipase, although the activity was weak. Previously, fruit extracts were also subjected to its inhibitory activity on lipase. Ghule et al reported that ethanol extract of *Lagenaria siceraria* fruit had good lipase inhibitory activity compared to other extractant solvents (methanol, ether, chloroform, ethyl acetate, n-butanol and water) (29). Therefore, it is possible that ethanol extract is considered as a good source for lipase inhibitors. To the best of our knowledge, the present study is the first report on the anti-lipase activity of *M. calabura* fruit.

The current study also demonstrated that *M. calabura* fruit had good antioxidant activity, rendering its action via different mechanisms, involving hydrogen atom or electron transfer mechanism. In addition, *M. calabura* fruit was shown to be able to chelate ferrous ions. It has been known that ferrous ions are considered to be the most effective pro-oxidant in biological system. Ferrous ions can take part in an electron transfer reaction that generate OH radicals through Fenton reaction ( $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \bullet OH$ ). The  $\bullet OH$  radicals can propagate lipid peroxidation by decomposing lipid hydroperoxides into its corresponding lipid radicals, such as peroxy and alkoxy radicals. In this case, chelating agent may act as a secondary antioxidant due to its activity of binding the catalysing Fe(II) ion. Therefore, chelating the iron(II) ions is an important property of antioxidant compound.

The antioxidant capacity of *M. calabura* fruit was further confirmed by its protective effect against DNA strand cleavage induced by  $\bullet OH$  radicals, generated through Fenton reaction. It has been reported that  $\bullet OH$

radicals react with nitrogenous DNA bases producing base radicals and sugar radicals. The base radicals in turn attack sugar moiety causing breakage of sugar phosphate backbone on nucleic acid, leading to DNA strand breakage (30). The present study demonstrated that the presence of fruit extract induced recovery of the damaged DNA. The effect is proved to be concentration-dependent. It is possible that the protective effect may be contributed to the radical scavenging activity against •OH radicals or Iron(II) chelating activity of the antioxidant compound in the extract.

The antioxidant activity of *M. calabura* fruit may be complementary to its excellent antidiabetic potential demonstrated in this study. Chronic hyperglycaemia condition as in diabetes mellitus induces the generation of oxidant and suppresses antioxidant defences system through multiple interacting enzymatic, nonenzymatic, and mitochondrial pathways, including protein kinase C (PKC), hexosamine, poly-ol, and AGE product formation pathways (31). This condition leads to oxidative stress and subsequent cell damage, which contributes to the pathophysiology of diabetes mellitus and its related complications such as diabetic retinopathy, neuropathy, and nephropathy. In particular, limited antioxidant defence due to autoimmune reactions causes damage to  $\beta$ -cells, resulted in impairment of insulin secretion. Therefore, dietary antioxidant intake may help protect cells from damages, thus may serve as a complementary strategy in prevention of diabetes and diabetic complications.

As the existing drugs have become ineffective, researchers have focused efforts in finding new antibacterial drugs. Plants are potential sources for novel and safe alternatives of pharmaceutically active compounds. Many researchers have studied the antibacterial activities of *M. calabura*. For example, Buhian et al reported the antibacterial activity of the leaf extract against *Aeromonas hydrophila* (8). The study reported that the activity was derived from the secondary metabolites contained in the leaves of *M. calabura*. In another, *M. calabura* leaf extract was bacteriostatic remedy against *A. hydrophila* which only inhibited bacterial growth up to 24 hours (MIC of 125  $\mu$ g/mL). It was suggested that the phenol compounds in *M. calabura* leaf extracts functioned as anti-bacterial agents (32). Several authors reported the ability of various extracts of *M. calabura* to inhibit the growth of certain bacteria. According to Sarojini and Maunika, methanol extracts of the leaves and fruits of *M. calabura* had antibacterial activities against *E. coli* and *Staphylococcus aureus* (33). Zakaria (2014) reported that methanol, aqueous and chloroform leaf extracts had antibacterial activities against *Corynebacterium diphtheria*, *S. aureus*, *Proteus vulgaris*, *S. epidermidis*, *Kasuria rhizophila*, *Shigella flexneri*, *E. coli*, *A. hydrophilia*, and *Salmonella typhi* (34). Despite various reports on the antibacterial activity of *M. calabura*, to the best of our knowledge, the present study is the first that

reported the anti-bacterial activities of *M. calabura* fruit against *C. violaceum* and *S. mutans*.

## Conclusion

The present study suggests that the ethanol extract of *M. calabura* fruit is an excellent inhibitor of  $\alpha$ -glucosidase and  $\alpha$ -amylase, which play important role in diabetes mellitus. The extract shows potential inhibition on xanthine oxidase and lipase. The study clearly demonstrated the antibacterial and the antioxidant activities of the fruit extract based on a variety of model systems *in vitro*. The present study confirms the ability of the extract to protect plasmid DNA against  $H_2O_2$  induced damage. Results suggest that *M. calabura* fruit might be further exploited for pharmacological ingredients for the development of future biomedicine or functional food, in particular for the treatment of chronic diseases associated with oxidative stress and infectious diseases. Future studies should be directed on the evaluation of bioactivities of *M. calabura* fruit in various *in vivo* systems and toxicity.

## Acknowledgement

We are grateful for the support from Institute for the Research and Community Service and the Faculty of Medicine and Health Sciences, Krida Wacana Christian University for research funding and the use of facilities.

## Authors' contributions

All authors (AS, AWS, IR, and KHT) contributed in the experimental studies, data acquisition, and data analysis. AS and KHT contributed to the concepts of ideas, experimental design, literature study, manuscript preparation and editing. AS did the manuscript review and submission. All authors read and agreed its publication.

## Conflict of interests

None to declare.

## Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or redundancy) have been completely observed by the authors.

## Funding and Support

This research was financially supported by the Research Institute of Krida Wacana Christian University (grant No. 13/UKKW/LPPM-FK/Lit/VIII/2018).

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