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Research Article

An assessment of *in vitro* antibacterial, anti-biofilm, anti-diabetic and antioxidant activities of bioactive compounds from *Muntingia calabura* Linn. leaves extracts

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ABSTRACT

Muntingia calabura Linn. is one of the medical plants that has been used to treat various types of illnesses. Thus, the aim of this experiment is to observe bioactive compounds from *Muntingia calabura* Linn. leaves extracts in different solvents such as boiled distilled water, 95% ethanol and 75% acetone. The results showed that bioactive activities of these leaves extracts were found from saponins, tannins, flavonoids, alkaloids, and steroids. For antibacterial result, the leaves were extracted with 95% ethanol (1:20) had the highest ability in inhibiting *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* DMST 4736 by agar well diffusion assay. The inhibition zones were 7.17±0.41 - 17.83±0.41 mm. Broth micro-dilution as MICs and MBCs revealed the MIC values as 12.50-50.00 mg/mL and the MBC values as 50.00-100.00 mg/mL, respectively. Moreover, crude extracts in different solvents also displayed inhibitory effects (>65%) on the biofilm formation. Particularly, the 95% ethanolic extract (1×MIC) had the highest inhibition of the biofilm formation in *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *S. typhimurium* ATCC 13311 as 90.03±0.28%, 88.05±0.24% and 86.25±0.25%, respectively. Based on antioxidant activities, the IC_{50} values of *Muntingia calabura* Linn. leaves extracts with 95% ethanol ranged between 106.60±0.30 - 293.97±0.10 µg/mL by DPPH assay. The total phenolics and flavonoids contents showed the highest as 258.95±0.64 mg GAE/g DW and 86.44±0.38 mg RE/g DW. In addition, it exhibited the most effective anti-diabetic activity in α-amylase inhibition (IC_{50}) as 98.67±0.15 µg/mL. The benefits of these results suggest that *M. calabura* Linn. leaves extracts can be used as a source of bioactive compounds in potential therapeutic agent for the bacterial infections as well as treatment of diabetes.

Keywords:
Bioactive compounds, Antibacterial activity, Antioxidant activity, Anti-diabetic activity, *Muntingia calabura* Linn.

1. INTRODUCTION

Medicinal plants have actually been used around the world for a long time without any reasonable knowledge of their pharmaceutical activities or active compounds. These medicinal plants have a significant advantage in treatment of many human diseases due to the various phytochemical constituents of all plant parts which have health benefits[1]. The secondary metabolites that are commonly found in plants are phenols, flavonoids, alkaloids, glycosides, saponins, terpenoids, steroids, tannins etc. Theses chemical constituents possesses diverse bioactivities such as enzyme inhibition, antioxidant, antimicrobial, anti-inflammatory activities etc[2]. Infectious disease is one of the biggest health concern. Most infections are caused by bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Enterococcus faecalis* which reported by the World Health Organization (WHO)[3]. These bacteria can form biofilms on the surface of medical devices and can cause respiratory, gastrointestinal and urinary tract infections. The first pharmacological choices to treat bacterial infections is antibiotics but, they had side effects. In fact, pathogenic bacteria have developed multi-drug resistance.

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It is difficult to treat bacterial infections in today. Therefore, plant-derived antimicrobial agents are more preferable to treat bacterial infections with fewer side effects. For the effect of antioxidants, plants are a good source of natural antioxidant activity. Antioxidants are chemical compounds that scavenge free radicals by either accepting or donating electrons to eliminate free radicals, resulting in a direct reduction in their ability to damage biological molecules. Free radicals are very harmful to human health which can cause several diseases in humans such as diabetes, cancer, atherosclerosis, cardiovascular, hypertension, hepatic disorders, alzheimer's disease, Parkinson etc[4].

In terms of diabetes, diabetes is considered to be one of the most challenging health problems of the 21st century. Diabetes is a chronic health problem and infections are more common and more severe in diabetic patients due to high blood sugar levels, resulting in abnormal functioning of the immune system[5]. Moreover, diabetic patients have an approximately 2-fold higher risk of bloodstream infections by various bacterial strains[6]. Alphaamylase and alpha-glucosidase inhibitors (acarbose and voglibose) are used to treat diabetes. Several studies have shown that inhibition of these enzymes is one of the most effective strategies for diabetes mellitus treatment[7-8]. Alpha-amylase is the first enzyme that converts starch into glucose. Thus, inhibition of this enzymes may help decline postprandial blood glucose levels[8-10]. However, both acarbose and voglibose exhibited some undesirable side effects, such as diarrhea, bloating and abdominal pain. The search for α-amylase inhibitors should thus increase and natural products as an alternative have received great attention.

Muntingia calabura Linn. (Jamaican cherry) is widely cultivated in the warmer areas of Asia (Malaysia, Indonesia, Philippines and Thailand) and belongs to the *Muntingiaceae* family. Many parts of this plant have been used to treat various types of illnesses. These leaves are widely used to treat gout, diabetes, flu symptoms, cancer, gastric ulcers and stomach[11-12]. *M. calabura* Linn. leaves mainly consists of several compounds, such as polyphenols, flavonoids, tannins, triterpenoids and saponins[13]. Several studies have shown that *M. calabura* leaves have certain pharmacological activities such as antinociceptive, anti-inflammatory, antimicrobial, antioxidants and anti-diabetic properties[10-14]. In addition to medicinal purposes, the leaves are also used as a tea-like beverage[11]. However, there are few existing reports of anti-diabetic activity by α-amylase inhibition of various solvent *M. calabura* Linn. leaves extracts as well as their antioxidant, antimicrobial and anti-biofilm activities. Thus, the current study aimed to screen the phytochemical compounds and also assess bioactivities of *M. calabura* Linn. leaves extracts in different solvents, particular their potential of antibacterial, anti-biofilm formation, antioxidant as well as anti-diabetic activities.

2. MATERIALS AND METHODS

2.1. Collection of plant materials and Preparation of plant extracts

Fresh leaves of *Muntingia calabura* Linn. were collected from Phraeksa Subdistrict, Mueang District, Samut Prakan Province, Thailand in November 2019. The plant samples were analyzed and classified by a botanical specialist and identified the specimen using the available literature[15]. The leaves were washed, cut into small size (estimate 1×1 cm) and dried in hot air oven at temperature of 40°C. The dried plants were ground into coarse powder and then macerated with different solvents in 95% ethanol (ratio=1:20) and 75% acetone (ratio=1:35) for 7 days under occasional shaking (room temperature) and boiled in distilled water (ratio=1:10) at temperature of 60°C for 60 min. After 7 days, the crude extracts in 95% ethanol and 75% acetone were filtered using Whatman No. 1 filter paper and centrifuged at 5,000 rpm for 15 min. The filtrate were concentrated using a rotary evaporator. Finally, the concentrated crude extracts were stored under refrigerated condition until used.

2.2. Qualitative phytochemical screening

Muntingia calabura Linn. extracts were carried out in standardized phytochemical tests (saponins, flavonoids, tannins, steroids, alkaloids, terpenoids, anthraquinones and cardiac glycosides) to assess the different bioactive constituents[16]. The results were observed from color change or precipitate formation after adding the specific reagents for 3-20 min.

2.3. Antibacterial activities

2.3.1. Preparation of bacterial inoculum

Bacteria strains used in this experiment were three Gram-positive bacteria such as *Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736 and *Staphylococcus aureus* ATCC 25923 and five Gram-negative bacteria such as *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212, and *Salmonella typhimurium* ATCC 13311. *Klebsiella pneumoniae* was the laboratory strain. All the microorganisms used in this study were obtained from the microbiology laboratory of the Biotechnology Department and were maintained on brain heart infusion (BHI, Difco) agar medium at temperature of 37°C.

2.3.2. Agar well diffusion method

The agar well diffusion assay was used to assess the antibacterial potential of plant samples. Sterile petri dish

plates containing 20 ml BHI agar were prepared. 100 µL of fresh culture suspensions (OD_{600}=0.5; ~10^8 CFU/mL) of indicator bacterial strains were inoculated in to BHI soft agar (5 mL; 0.75% agar) and then overlaid on BHI agar plates. After 10 min, sterile cork borer was used to make wells over the agar plates. Crude extract solutions (100 µL of 50, 100, 200 and 400 mg/mL) were added into each well. All plates were further incubated for 24 hours at temperature of 37°C. After incubation at 37°C for 24-48 hours, the diameter of inhibitory zones (IZ) around were measured in millimetres (mm). Ampicillin concentration 10 µg/mL used as positive control[17].

2.3.3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration values (MBC) assay

The minimum inhibitory concentration of plant extracts against indicator strains was determined by the broth micro-dilution method in 96 well plates[17]. The extracts were serially diluted in two-folds to obtain concentrations from 1,600 to 1.56 mg/mL. Then, 10 µL of the bacterial suspensions (OD_{600}=0.5; ~10^8 CFU/mL) was added to each well and incubated at temperature of 37°C for 24 hours. The first well that exhibits no turbidity is considered as MICs. Ampicillin concentration 10 µg/mL used as positive control.

For the MBC values, one loop from each well with no visible turbidity was streaked on BHI plates, and lowest concentration that showed no visible bacterial colonies on the freshly BHI agar plates was considered as MBCs.

2.4. Anti-biofilm activity

Biofilm inhibition was performed in sterile flat-bottomed 96-well microplate by modified crystal violet method[17]. 200 µL of BHI broth containing pathogenic strains (OD_{600}=0.5; ~10^8 CFU/mL) and 200 µL of each crude extract (1×MIC value) were added to the microplate. Microplates were then incubated at temperature of 37°C for 24 hours to culture the biofilms. Plates were washed with 200 µL PBS (phosphate buffer saline; pH 7.6) to remove medium and non-adherent bacteria. After washing, the biofilm formed was fixed with methanol for 15 min and then stained with 0.1% crystal violet (200 µL/well) for 15-20 min at room temperature. The excess staining solution was rinsed three times with sterile distilled water. Finally, the adherent cells were re-solubilized with 150 µL of 95% ethanol per well and the optical density (OD) of each well was measured at 570 nm versus the negative control group (bacteria+growth media). The percentage of biofilm inhibition was determined by following equation:

$$\text{Percentage (\%) of biofilm inhibition} = [(A_c - A_e)/A_c] \times 100 \quad (1)$$

where A_c is the absorbance of the control, A_e is the absorbance of the extract.

2.5. Antioxidant activities

2.5.1. Total phenolic content assay

The total phenolic content (TPC) was determined using Folin Ciocalteau method[18]. An aliquot of 100 µL of extracted solution was mixed with 750 µL of fresh Folin-Ciocalteu reagent (diluted with distilled water in 1:10 ratio). After 10 min, 750 µL of sodium carbonate (6% w/v; Na_2CO_3) was added to mix solution and then allowed to stand at ambient temperature for 90 min under the dark condition before measuring the absorbance at 725 nm by a spectrophotometer. The phenolic content in samples was derived from the standard calibration curve of the gallic acid (0.05-0.25 mg/mL). The TPC was expressed as mg gallic acid equivalent per g of dried weight (mg GAE/g DW).

2.5.2. Total flavonoid content assay

Total flavonoid content (TFC) of tested extracts was determined using the aluminum chloride assay[18]. An aliquot of 200 µL of crude extracts (of appropriate concentration) was mixed with 2.3 mL of 30% methanol solution and then added 100 µL of 0.5 M $NaNO_2$ and 100 µL of 0.3 M $AlCl_3$, respectively. The mixture solution was thoroughly mixed with vortex and left to stand for 5 minutes at ambient temperature in the dark before adding the 500 µL of 1M NaOH. Finally, the absorbance was taken against a blank without the $AlCl_3$ with same mixture at 506 nm using UV-spectrophotometer. The TFCs were calculated using standard calibration curve of the rutin (0.25-4.00 mg/mL). The result was expressed as mg of rutin equivalent per 100 g of dried weight (mg RE/g DW).

2.5.3. DPPH radical scavenging assay

The free radical scavenging activity of the fractions was measured *in vitro* by 2,2′ -diphenyl-1-picrylhydrazyl (DPPH) assay[18]. A 900 µL aliquot of 0.1 mM DPPH in methanolic solution was mixed with 100 µL of plant extracts dissolved with methanol (various concentrations 0.005-0.10 mg/mL). The reaction mixture were kept in the dark at ambient temperature for 15-30 min. The absorbance of mixture was then read at 517 nm. Ascorbic was used as the positive control. The antioxidant activity of the extract was assessed by calculating the percentage (%) of DPPH scavenging activity. The concentration of samples causing 50% inhibition of DPPH radical (IC_{50}; mg/mL) was determined graphically.

2.5.4. Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the previous method[18]. The fresh FRAP reagent (0.3 mM sodium acetate buffer (pH 3.6), 20 mM $FeCl_3$ and 10 mM of

ferric-tripyridyltriazine (TPTZ) in a ratio of 10:1:1 (v/v/v)) was warmed to 37°C in oven until used. A total of 300 µL of crude extracts was mixed with 2.7 mL of the FRAP reagent. After 30 min incubation, the absorbance was read at 596 nm using a spectrophotometer. Standard curve of ascorbic acid (0.01-0.08 mg/mL) was prepared. Results were expressed as mg of ascorbic acid equivalents/g fresh weight (mg AAE/DW) of the plant materials.

2.6. Anti-diabetic activity by *in vitro* α-amylase inhibition assay

The α-amylase inhibition activity of the extracts were carried out according to the reported method[19]. 500 µL acarbose (positive control) or plant extracts at different concentrations were incubated with 500 µL of α-amylase (1 UI/mL in 0.02 M sodium phosphate buffer; pH 6.9) at room temperature for 10 min. After 10 min, the mixture solution was added 1% (w/v) starch solution (500 µL in 0.02 M sodium phosphate buffer; pH 6.9) and incubated at 37°C for 20 min. Then, 1 mL of 3, 5-dinitrosalicylic acid (DNS) reagent was added before boiling for 5 min for stopping reaction. Next, the mixture was added 10 mL of distilled water. After cooling at ambient temperature, the absorbance was measured at 540 nm. A blank control was prepared as above without extract sample. The percentages of α-amylase inhibition were calculated using as following

$$\alpha\text{-amylase inhibition (\%)} = [A_c\text{-}A_e / A_c] \times 100 \quad (2)$$

where A_c is the absorbance of the control, A_e is the absorbance of the extract.

2.7. Statistical analysis

Values were recorded as mean±standard error of the mean. Statistical difference between the means was determined by one-way ANOVA followed by g Tukey's multiple comparisons or paired t-test (α=0.05) using Graphpad prism 9.0 software.

Table 1. Phytochemical screening of crude *Muntingia calabura* Linn. leaves extracts.

Phytochemical constituents	Test reagents	Distilled water	95%Ethanol	75%Acetone
Saponins	Froth formation test	+	-	-
Tannins	Ferric chloride test	+	-	-
Flavonoids	Shinoda test	+	+	+
	Lead acetate test	+	+	+
Anthraquinones	Borntrager's test	-	-	-
Steroids	Libermann test	+	+	+
	Keller-Kiliani Test	+	+	+
Terpenoids	Salkowski test	+	+	+
Cardiac Glycosides	Keller-Killiani test	-	-	-
	Kedde reagent	-	-	-
Alkaloids	Dragendoff's reagent	-	+	+
	Wangner's reagent	-	+	+

+: Presence; -: Absence

3. RESULTS AND DISCUSSION

3.1. Percentage of yield and phytochemical screening of *Muntingia calabura* Linn. extracts

The extraction yields of *Muntingia calabura* Linn. extracts in different solution such as boiled distilled water, 95% ethanol and 75% acetone varied from 1.87± 0.16 - 6.76±0.21% (w/v). Distilled water extraction produced the highest amount of all extractable compounds while the 95% ethanol extraction yield is the lowest when comparison with other solvents (75% acetone; 3.76% yield). The percentage of yields varied with solvent of extraction in this work which is consistent with study of Zheleva-Dimitrova et al. (2021)[20]. The phytochemical profiles of *Muntingia calabura* Linn. leaves (MCL) are presented in Table 1. The aqueous extract was contained the greatest number of secondary metabolite classes (5 out of 8 phytochemical compounds). Flavonoids, steroids and terpenoids were detected in all crude extracts of MCL. Meanwhile, anthraquinones and cardiac glycosides were found absence in all crude leave extracts. Saponins and tannins were present only in aqueous extract, while Buhian et al. (2016) has been reported the presence of saponins and tannins in ethanolic extract of *M. calabura* Linn. leaves[21]. Alkaloids were found in crude 95% ethanolic and 75% acetonic extract of MCL.

Similar to previous study of Situmorang et al. (2022) and Priya and Yasmin (2022) who reported that crude ethanol[22] and acetone[23] extracts of *Muntingia calabura* Linn. leaves contained flavonoids, terpenoids and steroids.

However, *M. calabura* Linn. leaves extract was reported to have many bioactive compounds, including saponins, tannins, flavonoids, alkaloids and catechins[24-26]. The presence of these compounds have also been reported to have antioxidant, antimicrobial, anti-inflammatory, antitumor activities[24,27]. The different chemical compounds of plants belonging to the same species may be

due to developmental stage, geographic regions, climate conditions and environmental factors (temperature, water stress, and light conditions)[27]. These phytochemical compounds are known to support bioactivity in medicinal plants. Hence this study designed an experiment to evaluate the bioactive properties of *Muntingia calabura* Linn. extract related to anti-diabetic activity, total phenolics and total flavonoids contents, antioxidant potential and inhibition of α-amylase.

3.2. Antibacterial activities

The antibacterial activities of the *M. calabura* Linn. leaves towards eight pathogenic strains by agar well diffusion were showed in Table 2. The result showed that crude extracts of *M. calabura* Linn. leaves in various solvents demonstrated varying degrees of antibacterial activities against various indicator bacterial strains. The assessment of antimicrobial activity via inhibition zone was interpreted as a moderate inhibition (6-9 mm); a strong inhibition (10-14 mm) and a very strong inhibition (>15 mm)[28]. All crude extracts of *M. calabura* Linn. leaves displayed inhibitory activity towards both Gram-positive and Gram-negative bacteria and revealed concentration-dependent towards all pathogenic strains. The antibacterial activities of the crude extracts were significantly higher against the all tested bacteria at concentration of 400 mg/mL ($p \leq 0.05$). Based on concentration of 400 mg/mL, 95% ethanolic extracts exhibited the highest bacteriostatic effect, following by 75% acetonic and aqueous extracts, respectively (Table 1). Interestingly, all tested concentrations of 95% ethanolic extract was

Table 2. Antimicrobial activity of crude *Muntingia calabura* Linn. leaves extracts by agar well diffusion method.

Concentration (mg/mL)		Zone of growth inhibition (∅ = 6 mm); mm±SD							
		Gram positive strains			Gram negative strains				
		BC	EF	SA	EC	KP	PA	PM	ST
Dw	**50**	8.00±0.00^{hi}	9.50±0.55^{gh}	7.50±0.55^{i}	-	9.00±0.00^{h}	10.55±0.55^{g}	8.50±0.55^{h}	-
	100	9.00±0.00^{h}	10.83±0.75^{fg}	8.00±0.00^{hi}	-	10.50±0.55^{g}	11.55±0.55^{f}	11.33±0.52^{f}	-
	200	11.67±0.82^{f}	14.83±0.75^{cd}	8.67±0.52^{h}	-	14.33±0.82^{c}	14.33±0.82^{c}	15.33±0.52^{c}	-
	400	13.17±0.41^{e}	17.33±0.82^{b}	10.17±0.41^{g}	-	16.67±0.52^{b}	16.55±0.52^{b}	17.50±0.55^{ab}	-
Eth	**50**	9.17±0.75^{h}	9.33±0.52^{h}	7.17±0.41^{i}	8.67±0.52^{h}	10.33±0.52^{fg}	8.67±0.52^{h}	10.33±0.52^{g}	10.33±0.52^{g}
	100	10.67±0.52^{g}	11.83±0.41^{f}	8.33±0.52^{h}	10.83±0.41^{fg}	13.00±0.63^{e}	10.50±0.55^{fg}	13.00±0.89^{e}	13.00±0.63^{e}
	200	11.83±0.98^{f}	15.17±0.75^{c}	8.50±0.55^{h}	13.67±0.82^{de}	13.33±0.52^{e}	13.50±0.55^{e}	14.67±0.52^{cd}	13.33±0.52^{e}
	400	13.67±0.82^{de}	16.50±0.55^{b}	10.33±0.72^{fg}	17.83±0.41^{a}	14.50±0.55^{cd}	14.50±0.55^{cd}	15.50±0.55^{c}	14.50±0.55^{cd}
Ace	**50**	-	-	-	-	9.00±0.00^{h}	-	-	-
	100	-	7.83±0.41^{i}	6.67±0.52^{j}	-	11.50±0.55^{f}	7.50±0.55^{i}	7.83±0.41^{hi}	-
	200	8.50±0.55^{hi}	9.50±0.55^{gh}	7.50±0.55^{i}	8.33±0.52^{hi}	12.67±0.52^{ef}	8.83±0.41^{hi}	9.67±0.52^{gh}	8.67±0.52^{h}
	400	11.00±0.63^{fg}	10.83±0.41^{fg}	8.50±0.55^{h}	9.83±0.41	14.67±0.52^{cd}	10.17±0.41$_{fg}$	10.17±0.41^{g}	11.50±0.55^{f}
Amp (10 µg)		20.17±1.38	23.17±0.98	26.50±0.55	19.50±0.55	14.17±1.17	25.33±0.58	21.67±0.58	24.00±0.00

^{abcdefghij} = values with different superscript differed significantly ($p \leq 0.05$) in different extracts; BC: *B. cereus* ATCC 1178, EF: *E. faecalis* DMST 4736, SA: *S. aureus* ATCC 25923, EC: *E. coli* ATCC 25922, KP: *K. pneumoniae*, PA: *P. aeruginosa* ATCC 27853, PM: *P. mirabilis* DMST 8212 and ST: *S. typhimurium* ATCC 13311; Dw: Distilled Water, Eth: 95% Ethanol, Ace: 75% Acetone; -: No zone of inhibition

potentially effective in inhibiting the all microbial growth while, 75% acetonic extract showed antimicrobial activity against all bacterial strains at plant concentrations of 200 and 400 mg/mL. 95% ethanolic extract of MCL displayed strong antagonistic activity against all pathogenic strains with inhibition zones (IZ) of 10.50±0.55-17.83±0.41 mm (100-400 mg/mL), while aqueous extract showed the best antagonistic effect to 6 indicator strains as *B. cereus* ATCC 1178, *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *K. pneumoniae*, *P. aeruginosa* ATCC 27853 and *P. mirabilis* DMST 8212 with IZ of 10.17±0.41 - 17.50±0.55 mm at 400 mg/mL. Similar to a study of Cheong et al. (2022) who reported that *M. calabura* Linn. extract had antimicrobial activity against *S. aureus* (IZ=14.33 mm)[26]. Moreover, 75% acetonic extract (at concentration 400 mg/mL) revealed strong antimicrobial activity towards eight pathogens but, it had moderate inhibitory effect against *S. aureus* ATCC 25923 (IZ=8.50±0.55 mm) and *E. coli* ATCC 25922 (IZ=9.83±0.41 mm) growths. Among indicator strains, *P. mirabilis* DMST 8212, *E. coli* ATCC 25922, *K. pneumoniae* were the most susceptible stains to crude aqueous, 95% ethanolic and 75% acetonic extracts, respectively, whereas *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 as gram-negative bacteria exhibited resistance to crude aqueous extract of MCL in all concentrations when compared with gram-positive bacteria (*B. cereus* ATCC 1178, *E. faecalis* DMST 4736 and *S. aureus* ATCC 25923) due to the composition of gram-negative bacteria with more complex structures than gram-positive organisms. A similar study by Cheong et al. (2022) has been reported that this plant extract had no inhibitory effect to the growth of *S. typhimurium*[26].

According to MIC and MBC assays, the antibacterial activities of the MCL extracts by broth micro-dilution assay are given in Tables 3. The result showed that all extracts displayed antimicrobial activities as shown the MIC values ranging from 12.50 to 400 mg/mL and MBC values ranging from 50 to 800 mg/mL. The MICs and MBCs values of ampicillin (positive control) were showed range from 0.078-1.25 mg/mL and 0.315-2.50 mg/mL for

Table 3. MICs and MBCs of crude *Muntingia calabura* Linn. leaves extracts against pathogenic strains by broth micro-dilution assay.

Pathogenic strains	Distilled water		95%Ethanol		75%Acetone	
	MICs (mg/mL)	MBCs (mg/mL)	MICs (mg/mL)	MBCs (mg/mL)	MICs (mg/mL)	MBCs (mg/mL)
Gram positive strains						
***B. cereus* DMST 5040**	100±0.00^{d}	200±0.00^{C}	50±0.00^{c}	200±0.00^{C}	400±0.00^{f}	800±0.00^{E}
***E. faecalis* DMST 4736**	400±0.00^{f}	800±0.00^{E}	25±0.00^{b}	100±0.00^{B}	200±0.00^{e}	400±0.00^{D}
***S. aureus* ATCC 25923**	100±0.00^{d}	200±0.00^{C}	12.50±0.00^{a}	50±0.00^{A}	400±0.00^{f}	800±0.00^{E}
Gram negative strains						
***E. coli* ATCC 25922**	400±0.00^{f}	800±0.00^{E}	50±0.00^{c}	100±0.00^{B}	200±0.00^{e}	400±0.00^{D}
K. pneumoniae	400±0.00^{f}	800±0.00^{E}	100±0.00^{d}	200±0.00^{C}	200±0.00^{e}	400±0.00^{D}
***P. aeruginosa* ATCC 27853**	400±0.00^{f}	800±0.00^{E}	50±0.00^{c}	100±0.00^{B}	200±0.00^{e}	400±0.00^{D}
***P. mirabilis* DMST 8212**	400±0.00^{f}	800±0.00^{E}	100±0.00^{d}	200±0.00^{C}	400±0.00^{f}	800±0.00^{E}
***S. typhimurium* ATCC 13311**	400±0.00^{f}	800±0.00^{E}	50±0.00^{c}	100±0.00^{B}	200±0.00^{e}	400±0.00^{D}

MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration
^{abcdef}=values in the MIC determination with different superscript differed significantly ($p \leq 0.05$)
^{ABCDE}=values in the MBC determination with different superscript differed significantly ($p \leq 0.05$)

all pathogenic strains. The crude 95% ethanolic extract of MCL gave notable antibacterial activity against all pathogenic strains with MIC values ranging from 12.50 to 100 mg/mL and MBC values ranging from 50 to 200 mg/mL. Interestingly, 95% ethanolic extract was also showed antimicrobial activity against *S. aureus* ATCC 25923 with MIC values of 12.50 and MBC values of 50 mg/mL. Crude aqueous extract had the best antagonistic activity values against *B. cereus* DMST 5040 and *S. aureus* ATCC 25923 (MIC value of 100 mg/mL and MBC value of 200 mg/mL). Furthermore, 75% acetonic extract showed antimicrobial activities towards all tested bacteria with MIC values of 200 mg/mL and MBC values of 400 mg/mL, excepted *B. cereus* DMST 5040, *S. aureus* ATCC 25923 and *P. mirabilis* DMST 8212 (MIC value of 400 mg/mL and MBC value of 800 mg/mL). According to a previous study on *M. calabura* Linn. leaves, the antimicrobial activity conducted by Sufian et al. (2013) revealed that ethanolic extracts against *S. aureus* (MICs and MBCs ranging from 1,250 to 2,000 μg/mL) but, it was not active against *B. cereus*, *E. coli* and *P. aeruginosa* at the highest concentration tested (MICs and MBCs of 5,000 μg/mL)[29]. Other study done by Cheong et al. (2022) who also reported that *methanolic M. calabura* Linn. extract had antimicrobial activity against *S. aureus* (MIC values of 7.81 mg/mL and MBC values of 15.63 mg/mL)[26]. Based on the ratio MBCs/MICs, all crude extracts of MCL had bacteriotic action towards all pathogenic strains due to their ratio MBC/MIC (ratio MBC/MIC≤4 considered as bactericidal and >4 was defined as bacteriostatic[30]. Although the MICs and MBCs values of crude aqueous and 75% acetonic extracts of MCL were very high, the 95% ethanolic extract of MCL had lower MICs and MBCs values than other extracts (Table 3). Therefore, 95% ethanolic extract is most suitable to be developed as an antimicrobial agent, especially *S. aureus* ATCC 25923 and *E. faecalis* DMST 4736 (MICs of 25 mg/mL and MBCs of 50 mg/mL).

According to anti-*E. coli* ATCC 25922 and -*S. typhimurium* activities of crude aqueous extracts, antibacterial activity by agar diffusion and broth micro-dilution methods (MICs of 400 mg/mL) was inconsistent (Table 2 and 3). These results did not show antibacterial activity against *E. coli* ATCC 25922 and *S. typhimurium* at a concentration of 400 mg/mL of aqueous MCL extracts by agar diffusion method due to the diffusion of the extracts and thickness of the agar medium. The agar diffusion assay thus is unacceptable and cannot be used to determine the MIC values of plant extracts due to its insensitivity. Lack of diffusion of nonpolar molecules to aqueous agar matrix[31].

3.3. Anti-biofilm activity

Microbial biofilms are an important virulence factor due to their abilities to tolerate antibiotics and avoid the host immune system[32]. Before evaluating the effect of the extracts on bacteria biofilm formation, the antimicrobial activity of the extract was first measured before assessment of the effect of plant extract on biofilm formation. At 1×MIC concentrations of crude extracts of MCL was used for evaluating the anti-biofilm activity. The anti-biofilm activity of *M. calabura* Linn. extracts against pathogenic bacteria was assessed by crystal violet staining assay and is demonstrated in Figure 1. All crude extracts showed good biofilm inhibiting activity towards bacterial strains within 24 hours after biofilm formation (65.22± 0.24 - 90.03±0.28%; $p \leq 0.05$) at 1×MIC concentration. Crude 95% ethanolic extract significantly showed the best anti-biofilm activity up to 90% ($p \leq 0.05$). Crude aqueous and 75% acetonic extracts displayed good inhibitory activity against tested bacterias with >65% of inhibiting biofilm formation. 95% ethanolic and aqueous extracts were the most effective in the inhibition of biofilm-forming of *S. aureus* ATCC 25923 with 90.03±0.28% (IZ of 7.00±0.00 mm at 1×MIC of 12.50 mg/mL) and 76.06±0.187% (IZ of 8.00±0.00 mm at 1×MIC of 100 mg/mL), respectively, whereas was *P. aeruginosa* ATCC 27853 (76.14±0.29%;

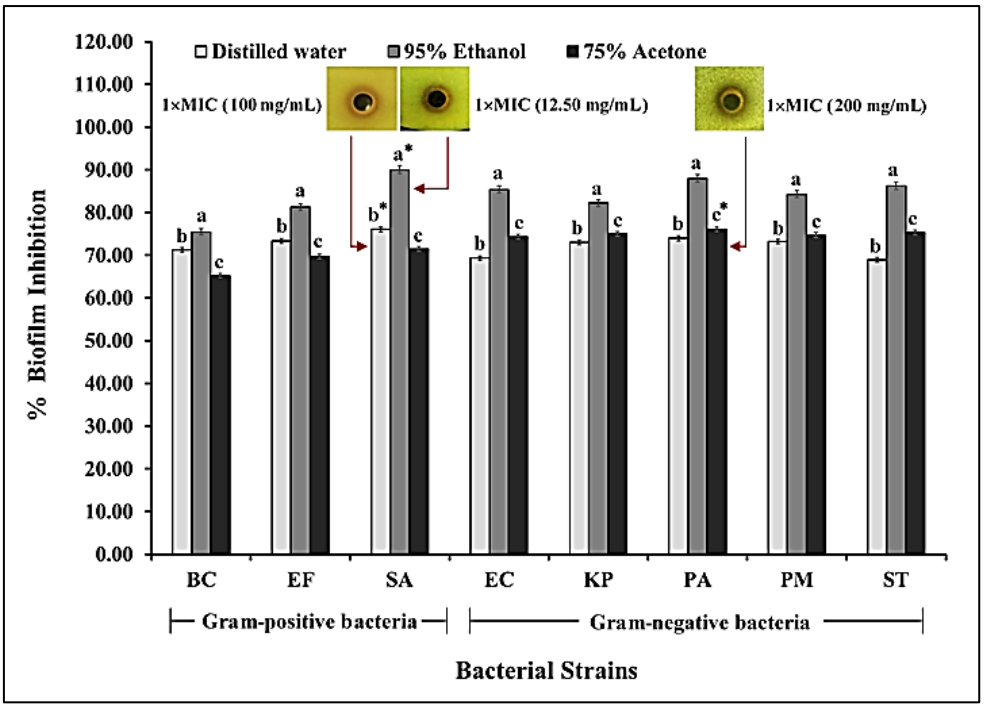

Figure 1. Biofilm inhibition percentage of crude *Muntingia calabura* Linn. leaves extracts (1×MIC) against pathogenic strains ([abc]: different superscript are significantly different in the same pathogenic strains ($p \leq 0.05$) and *: significantly different ($p < 0.05$) when compared with in the same extracts).

IZ of 8.83±0.41 mm at 1×MIC of 200 mg/mL) by 75% acetone extract (Figure 1). The bacteria *P. aeruginosa* is a well-known biofilm producer and its biofilm cause the acute and chronic infections (excretory and respiratory systems)[33]. The lowest anti-biofilm activity was obtained against *B. cereus* ATCC 1178 (65.22±0.24%) in 95% ethanolic and 75% acetonic extracts and *S. typhimurium* ATCC 13311 (68.96±0.28%) in aqueous extract. The study of MosaChristas et al. (2022) showed that *M. calabura* methanol leaves extracts were most potent anti-biofilm formation of *P. aeruginosa*[34]. *Enterococcus faecalis* is a common biofilm former usually implicated in urinary tract infections, wound infections, and dental infections[35]. All extracts of *M. calabura* leaves had the good anti-biofilm potential against the *E. faecalis* DMST 4736 biofilm (69.72±0.054 - 81.29±0.33 % of biofilm inhibition). Based on antimicrobial and anti-biofilm activities, the current study clearly indicated *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 as pathogenic strains were shown to be highly susceptible to all crude MCL extracts, especially 95% ethanolic fraction. Although there are the numerous studies on the antimicrobial activity of *M. calabura* leaves extracts, there are no clear assessment of their biofilm-forming inhibition.

3.4. Total phenolics and Flavonoids contents

Phenolic compounds are considered to be the main phytochemical compounds that play an antioxidant role of plant materials due to their hydroxyl groups[36]. The results of total phenolic content (TPC) and total flavonoid (TFC) contents were summarized in Table 4. The result showed that the 95% ethanolic extract of MCL significantly displayed the highest TPC of 258.95±0.64 mg GAE/g DW and TFC of 86.44±0.38 mg RE/g DW, following by crude aqueous and 75% acetonic extracts, respectively (Table 4; $p \leq 0.05$). Generally, the more polar solvent influences the amount of TPC which gives a higher value which is consistent with this study[36].

Flavonoids are considered to be one of the most

Table 4. Antioxidant and anti-α-amylase activities of crude *Muntingia calabura* Linn. leaves extracts.

Samples	Antioxidant activities					IC_{50} of Anti-α-amylase activities (µg/mL)
	TPCs (mg GAE/g DW)	TFCs (mg RE/g DW)	DPPH assay % Inhibition		FRAP assay IC_{50} (µg/mL)	
MCD	109.24±0.93[b]	67.85±0.34[b]	71.45±2.36[c]	539.80±0.10[c]	67.40±0.28[b]	249.00±0.30[b]
MCE	258.95±0.64[a]	86.44±0.38[a]	87.46±1.41[a]	106.60±0.30[a]	127.69±0.80[a]	98.67±0.15[a]
MCA	35.70±0.16[c]	4.69±0.24[c]	79.93±1.43[b]	293.97±0.10[b]	68.67±0.62[b]	272.39±0.25[c]
Ascorbic	-	-	-	16.80±0.10	-	83.60±0.31

[abc] = different superscript are significantly different from each other ($p \leq 0.05$)
MCD = *M. calabura* extract in distilled water; MCE = *M. calabura* extract in 95% ethanol; MCA = *M. calabura* extract in 75% acetone

prevalent groups of natural constituents found in plants[36]. Total flavonoids content (TFC) was estimated by using aluminum chloride reagent. Total flavonoids content in crude *Muntingia calabura* Linn. extracts was solvent dependent with ranging from 4.69±0.24 to 86.44±0.38 mg RE/g DW. The greatest TFC was significantly observed with crude ethanolic extract of MCL (86.44±0.38 mg RE/g DW), following by crude aqueous (67.85±0.34 mg RE/g DW) and 75% acetonic (4.69±0.24 mg RE/g DW) extracts, respectively ($p \leq 0.05$). According to Kodama et al. (2010), plants with high phenolic and flavonoid contents have significant antioxidant potential[37] which supported the TPC and TFC results of *Muntingia calabura* Linn. leaves extracts (ethanol > aqueous > acetone) in present study. Based on total flavonoids contents of various ethanol concentration of *Muntingia calabura* Linn. leaves extracts (96% v/v; 70% v/v and 50% v/v) by Pertiwi et al. (2020), the 96% ethanolic extract showed the highest total flavonoids content with 3,387 ug QE/100 g[38]. Flavonoids are believed to decline blood glucose levels by enhancing insulin secretion and reducing insulin resistance[39].

3.5. Antioxidant activities

3.5.1. DPPH radical scavenging activity

The antioxidant capacity of the crude extract of the *M. calabura* leaves was evaluated by DPPH and FRAP assay. In the DPPH assay, the highest IC_{50} value means the lowest antioxidant activity. 95% ethanolic extract (IC_{50}=106.60±0.30 μg/mL) significantly showed higher activity than the 75% acetonic (IC_{50}=293.97±0.10 μg/mL) and aqueous (IC_{50}=539.80±0.10 μg/mL) extracts, respectively (Table 4; $p \leq 0.05$). A study done by Sindhe et al. (2013)[40] who reported that ethanolic (IC_{50} value of 79.96± 0.91 μg/mL and aqueous (IC_{50} value of 97.638±2.06 μg/mL) extracts of *M. calabura* leaves had effective antioxidants than same plant extracts in this study. In a work carried out by Balakrishnan et al. (2011) reported that hydroethanolic extract showed DPPH scavenging activity with the IC_{50} value of 8.5 μg/mL[41]. As for the radical scavenging activity (%), All *M. calabura* leaves extract showed the radical scavenging activity ranging between 71.45±2.36 - 87.46±1.41%. Aqueous extract of MCL had DPPH radical scavenging activity with 71.45±2.36% while, aqueous extract showed 85.7-89.0% of the radical scavenging activity by Zakaria et al. (2011)[42]. Plants with high phenolic and flavonoid contents have significant antioxidant potential which is consistent with this study.

3.5.2. Ferric-reducing antioxidant power (FRAP) assay

95% ethanolic extract expressed the greatest antioxidant power with 127.69±0.80 mg AAE/g DW, while aqueous extract exhibited the lowest antioxidant power with 67.40±0.28 mg AAE/g DW (Table 4). The study of Jisha et al. (2019) demonstrated that aqueous extracts of *M. calabura* leaves (petroleum ether, chloroform extract, ethyl acetate, methanol and aqueous extracts had potent reduction of ferric iron (IC_{50} value of 82.42±0.03 - 247.71± 0.03 μg/mL)[43]. Although the antioxidant potential of MCL was be lower than that of positive control (ascorbic acid) in DPPH and FRAP assays, this study revealed that 95% ethanolic extract had prominent antioxidant activity due to the presence of phenolic compounds (containing phenolic hydroxyls). Thus, 95% ethanolic extract of MCL could be attributable to the observed high antioxidant properties of phenolic compounds.

In the current study, phenolic and flavonoid compounds were the important secondary metabolites of the MCL extracts which showed antibacterial and antioxidant potential (Table 3 and Table 4). The 95% ethanololic extract of MCL had the highest antibacterial and antioxidant activities. These might be due to the greater total phenolics and flavonoids contents found in 95% ethanolic extract.

3.6. Anti-diabetic activity

The present study investigated the antidiabetic activity of *Muntingia calabura* Linn. leaves extracts by evaluating their inhibitory activity against α-amylase. Inhibition of α-amylase contributes to improve symptoms of type 2 diabetes (controlling blood glucose levels in the blood) by slowing starch hydrolysis and delaying glucose absorption patients[9]. The α-amylase is one of targets to block carbohydrate degradation into absorbable monosaccharides, thereby reducing the postprandial hyperglycemia[7]. The results showed that all crude extracts of MCL demonstrated lower antidiabetic potential than the standard (Figure 2). The 95% ethanolic, aqueous and 75% acetonic extracts at a concentration 100 μg/mL showed a percentage inhibition of 50.76±0.15, 39.63±0.34 and 36.08± 0.37, respectively (Aacarbose=59.81±0.43%). However, highest anti-α-amylase activity of MCL extracts was significantly recorded in 95% ethanolic fraction (IC_{50}= 98.67±0.15 μg/mL), followed by aqueous (IC_{50}=249.00±0.30 μg/mL) and 75% acetonic (IC_{50}=272.39±0.25 μg/mL) extracts, respectively (IC_{50} of acarbose=83.60±0.15 μg/mL; $p \leq 0.05$). Phenolic compounds in plant extracts have antidiabetic effects in plant extracts because, phenolic compounds are one of the potent inhibitors of α-amylase[44]. These results are in complete agreement with the total phenolic contents found in the *M. calabura* leaves extract (95% ethanol > aqueous > 75% acetone; Table 4). The study of Zolkeflee et al. (2022) have been reported that the 50% ethanolic extract of *M. calabura* leaves, exhibited the highest α-amylase inhibitory activity with IC_{50} values of 26.39±3.93 μg/mL[10].

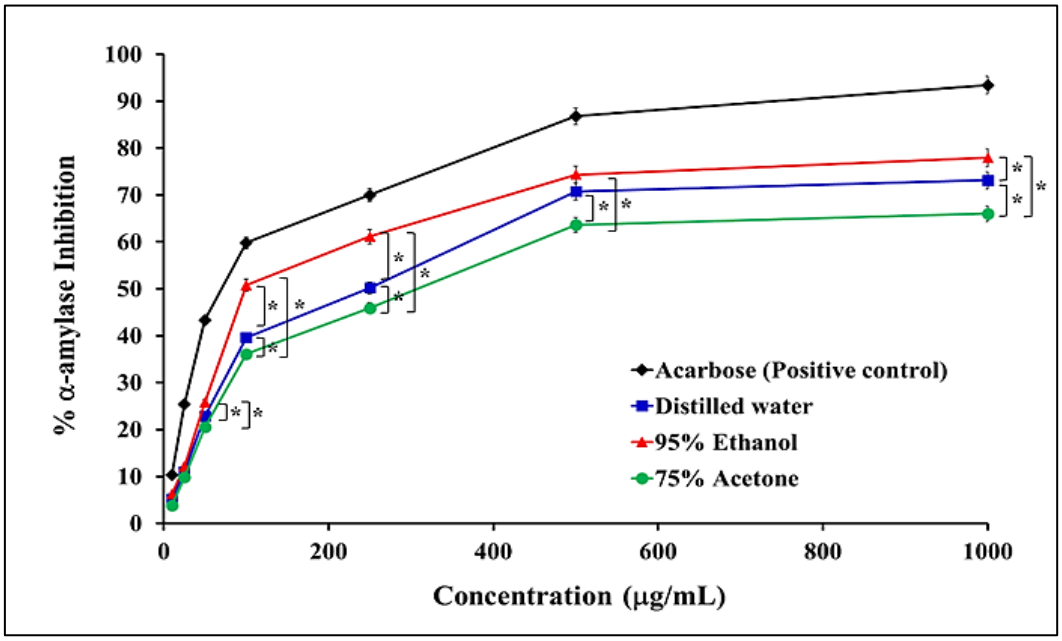

Figure 2. Anti α-amylase activity of crude *Muntingia calabura* Linn. extracts and acarbose (*=$p \leq 0.05$)

4. CONCLUSION

The current findings indicated that *Muntingia calabura* Linn. leaves extracts contained various bioactive compounds, including saponins, tannins, flavonoids, alkaloids and steroids which exhibited strong antimicrobial and anti-biofilm formation activities against various pathogenic strains, especially *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. faecalis* DMST 4736. The 95% ethanol was a suitable solvent for the extraction of an important phytochemical compounds due to its good bioactivity in this study. The 95% ethanolic extract of *M. Calabura* Linn. leaves extracts showed the highest total phenolics and flavonoids contents and had also the greatest potency of antioxidant activities by DPPH and FRAP assays. Moreover, ethanolic extract of *M. calabura* Linn. leaves were the most potent anti-diabetic activity by inhibiting α-amylase. However, further studies are needed to evaluate *in vivo* animal model and purify bioactive compounds to develop effective antimicrobial agents and anti-diabetic drugs without undesired side effects.

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