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Original Article

α-Glucosidase and α-amylase inhibitory effects with anti-oxidative activity of *Tetracera loureiri* (Finet & Gagnep.) Pierre ex Craib leaf extracts

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ABSTRACT

Tetracera loureiri (Finet & Gagnep.) Pierre ex Craib leaf extracts were evaluated the inhibitory effect of diabetes mellitus (DM)-related enzymes including α-glucosidase and α-amylase. The high polarity extracts, methanol-(TTL1) and aqueous-methanol-(TTL4) extracts, displayed higher potency than the nonpolar and low polarity extracts (hexane- and dichloromethane-extracts). Both extracts also expressed enzyme inhibitory activity stronger than those of positive control, acarbose. Additionally, they also exhibited good antioxidative effects conducted from DPPH radical scavenging activity and ferric reducing power. The antioxidant effects of both polar extracts were comparable to those of positive controls, quercetin and Trolox. These antioxidative properties have been expected to synergize antidiabetic activities by preventing DM complications. Therefore, the prominent bioactivities of the polar extracts, TTL1 and TTL4, are attractive to further isolate the active components for antidiabetic agent development.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that has continuously increased incident rates for more than three decades. World Health Organization (WHO) reported the incidence of DM patient reach to 422 million cases worldwide in 2014. There were 1.5 million deaths caused by DM and 2.2 million deaths from other diseases related to high blood glucose in 2012[1]. The high blood glucose is one important indicator of the disease progression, therefore, reducing the glucose level is a crucial mechanism for diabetes treatment[2]. Several molecular targets have been proposed for anti-hyperglycemic drug development including α-glucosidase[3] and α-amylase enzyme[4]. These enzymes are required for complex sugars digestion prior monosaccharide absorption. Inhibition of α-glucosidase leads to delay glucose absorption on brush border in the small intestine while α-amylase inhibitors slow glucose ingestion of pancreatic amylase[3-5]. Presently, there are the approved anti-hyperglycemic drugs, acarbose and miglitol, which inhibiting these enzyme functions.

Plants in genus *Tetracera* sp. have been shown potential to be an antidiabetic agent including *Tetracera indica* Merr.[6] and *Tetracera scandens* Linn. Merr.[7]. Both species have been used as a folk medicine for DM treatment in Malaysia. Aqueous and methanol extracts of *T. indica* have been reported to reduce the blood glucose in alloxan-induced diabetic rats with no hypoglycemic effect to normal rats[6]. *T. scandens* aqueous and methanol leaves extracts reduced fasting blood glucose level in diabetic rats without hypoglycemic effect[7]. Mechanism of action of isolated compounds of *T. scandens* has been evaluated to be muscle glucose uptake induction[8].

Tetracera loureiri (Finet & Gagnep.) Pierre ex Craib or "Rot Su Khon Khao" in Thailand is a woody climber with white aroma flower located in the Southeast Asia area[9]. The roots grinding with water has been traditionally used for lymphoma treatment by drinking or rubbing on the skin in Udonthani province[10]. A decoction of *T. loureiri* roots and *Eulophia bicallosa* (D.Don) Hunt& Summerh. was used in traditional medicine for relieving muscle pain[11]. Although *T. loureiri* has been used in folk medicine for a long time, limited data supported the pharmacological activities. In 2003, the ethanolic extract of *T. loureiri* stems has been reported the hepatoprotective activity in mice treated with high dose of paracetamol or tertiary-butylhydroperoxide. Meanwhile, the extract also exhibited a strong antioxidative activity, with EC_{50} 2.1 ± 0.2 µg/mL, using DPPH radical scavenging assay[12]. *T. loureiri* extract also expressed a good inhibitory effect to tyrosinase enzyme at IC_{50} 0.202 mg/mL with low toxicity to melanocyte (B16-F10). Besides, the extract also inhibited melanin production in B16-F10 cells with the same potency as those of positive control, kojic acid[13].

In this study, we evaluated the α-glucosidase and α-amylase inhibitory effect of the *T. loureiri* extracts, which there was no previous report of these inhibitory activities. In addition, antioxidative activities of the extracts were also investigated. The antioxidants prevent the oxidative stress and advanced glycation end products formation which are the risks in DM complication. Therefore, the antioxidants have a synergistic effect of the anti-diabetic activities[14].

2. MATERIALS AND METHODS

2.1. Materials

The leaves of *Tetracera loureiri* were collected from Thammasat University, Rangsit campus, Thailand in September 2017. The plant was authenticated by Mr.Bordintorn Sonsupab, Sirinthon Plant Herbarium Museum, Department of Agriculture. The voucher specimen (no. BK 070799) was deposited at the Bangkok Herbarium, Department of Agriculture, Bangkok, Thailand.

Extracting solvents (hexanes, dichloromethane, and methanol) used in this work were purchased from Honeywell (Germany). α-Glucosidase enzyme type I from *Saccharomyces cerevisiae* and α-amylase type VI-B from porcine pancreases, *p*-Nitrophenyl α-D-glucopyranoside, acarbose, gallic acid, quercetin hydrate, 2, 4, 6-tripyridyl-s-triazine (TPTZ), 2,2-Diphenyl-1-picryl hydrazyl (DPPH) and other chemical substances were purchased from Sigma-Aldrich (USA).

2.2. Preparation of plant extracts

The *T. loureiri* dried leaf powder (1 kg) was exhaustively extracted in methanol (5×3 Liters) using conventional maceration technique at room temperature. The methanol extracted fluid was evaporated under reduced pressure to obtain the methanolic crude extract (120.5 g). An aliquot of methanol extract (23.0 g) was further separated through a consecutive partition to yield hexane-, dichloromethane- and 60% methanol in water-extracts (1.2, 1.3 and 15.9 g, respectively). All fractions were dried using a rotary evaporator and kept in a desiccator for bioassay testing.

2.3. Phytochemical analysis of *T. loureiri* extracts

Preliminary phytochemical screening of *T. loureiri* extracts was performed according to the method described by Aiyegoro and Okoh (2010)[15]. The plant extract was individually prepared in methanol to produce 10 mg/mL solution. A small piece of magnesium ribbon was added into the extract solution followed by 2-3 drops of concentrated hydrochloric acid (Shinoda's test). The presence of a red or an orange color solution indicated the existing of flavonoids. Steroids or triterpenoids components were determined using Salkowski's test. Concentrated sulfuric acid was carefully added

into the plant extract solution (10 mg/mL in dichloromethane). A reddish brown color indicated the existing of steroids while a yellow color represented the triterpenoids substances[16]. *T. loureiri* extracts were tested with ferric chloride reagent and 1% (w/w) gelatin solution to investigate phenolic moiety and tannin substances. A few drops of ferric chloride reagent were added into the aqueous solution (10 mg/mL) of each plant extract. A dark blue coloration was produced in the presence of phenolic components. To investigate protein precipitation ability of tannins, the gelatin solution was added to the aqueous extract solution. The positive result showed white precipitate in the extract solution. For alkaloids detection, the plant extract was dissolved in methanol (10 mg/mL) and then a few drops of Dragendorff's reagent was added into each extract solution. The orange-red precipitates were formed by complexation of metal ion of the reagent and alkaloids.

2.4. α-Glucosidase inhibitory assay

The inhibition of α-glucosidase activity protocol was performed using a modified method of Kim et al. in 2005[17]. The plant extract was individually dissolved in 0.1 M sodium phosphate buffer (pH 6.8) to obtain the extract solution with final concentration in a range of 0.50-30 µg/mL. 50 µl of sample solution was added into 96-well plate containing 50 µl of 1 mM *p*-nitrophenyl-α-D-glucopyranoside. The sample mixtures were left at 37°C for 5 min and then 50 µl of 0.05 U/ml of α -glucosidase enzyme in buffer was added to each well. The reaction was further incubated at 37°C for 30 min. After incubation period, the reaction was stopped using 50 µl of 0.3 M sodium carbonate solution. The absorption of the reaction mixture was observed at wavelength 400 nm using microplate reader (BioRAD Benchmark PlusTM, USA). Percentage of enzyme inhibitory effect and IC_{50} were calculated and compared to the result of the α-glucosidase inhibitor, acarbose.

2.5. α-Amylase inhibition assay

The α-amylase inhibition procedure was modified from the previous protocol of Bernfeld (1955)[18]. The plant extracts were prepared in phosphate buffer (pH 6.9) at concentration 50 µg/mL. 400 µL of plant sample was mixed to 400 µL of 1% (w/v) starch in phosphate buffer solution. The mixture was incubated at 37°C for 5 min. The 400 µL of α-amylase (0.625 U/mL in phosphate buffer, pH 6.9) was then added to the mixture and incubated at 37°C for 5 min. Addition of 800 µl of DNS reagent (a mixture of 3,5-dinitrosalicylic acid and sodium potassium tartate in a ratio of 1:30 in 0.4 M NaOH) was performed. The reaction was terminated by boiling at 100°C for 10 min. The reaction mixture was left to room temperature and was diluted with ultrapure water before transfer into 96-well plate. The absorption at wavelength 540 nm was detected using microplate reader (BioRAD Benchmark PlusTM, USA). The percentage of inhibition and IC_{50} were calculated. Acarbose was used as a positive control.

2.6. DPPH radical scavenging activity assay

The DPPH (2,2-Diphenyl-1-picryl hydrazyl) radical scavenging activity was determined following the modified method of Bran-Williams et al. (1995)[19]. One hundred microliter of the plant extract and quercetin hydrate standard solutions were individually placed in a 96-well plate followed by addition of 100 µl DPPH solution (200 µM in ethanol). The mixture was protected from light and left at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using the microplate reader (BioRAD Benchmark PlusTM, USA). The scavenging activity was reported as half-maximal inhibitory concentration (IC_{50}).

2.7. Ferric Reducing Antioxidant Power (FRAP) assay

The ferric reducing antioxidant power of *T.loureiri* extract was investigated following to the modified protocol of Benzie and Strain's method[20]. The FRAP solution was freshly prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3.6H_2O$. The FRAP solution was mixed and incubated at 37 °C for 30 min prior to use. Twenty microliter of each of plant extracts and standard solution (Trolox) were placed in a 96-well micro-titer plate. Then, FRAP solution (180 µl) was added to both extracts and standard wells, kept for 30 min in darkness. For this experiment,

the antioxidative activity was determined from reduction reaction of Fe^{3+} of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex (yellow) to Fe^{2+} of ferrous (blue) form. Development of ferrous tripyridyltriazine complex was measured by absorbance at 593 nm using microplate reader (BioRAD Benchmark PlusTM, USA). The reducing antioxidant power were expressed as μM Trolox equivalent per gram extract (μM TE/g of extract) and half-maximal inhibitory concentration (IC_{50}).

3. RESULTS AND DISCUSSION

3.1. Preparation of *T. loureiri* extracts

The methanolic extract (TTL1) 120.5 g was obtained from 1 kg of *T.loureiri* dried leaves powder yielding 12.0% (w/w). The consequence liquid-liquid partition of methanol extract (23.0 g) gave three fractions including hexane-(TTL2), dichloromethane-(TTL3) and aqueous-methanol (TTL4) extracts as 1.3, 1.2 and 15.9 g, respectively. The percent yields of the polar extract (TTL4) provided the highest yield as 69.1% (w/w). The remaining extracts, TTL2 and TTL3, were yielded in the same range as 5.2% (w/w) and 5.6% (w/w), respectively.

3.2. Phytochemical screening of *T. loureiri* extracts

In order to estimate the chemical constituents of *T. loureiri* leaf extracts, the phytochemical tests were performed. Four groups of secondary metabolites were determined including flavonoids, tannins, terpenoids/steroids and alkaloids. The results of all testing are shown in Table 1. The high polar extracts including TTL1 and TTL4 exhibited the similar positive results. They gave the positive red color, dark-blue color, and gelatin precipitation with Shinoda's, ferric chloride and gelatin test, respectively, which referred to flavonoids, phenolic compounds and tannins containing in the extracts. The reddish-brown color with Salkowski's test indicating the presence of steroids in TTL1, TTL3 and TTL4. Only TTL4 showed less intense reddish-brown color due to the steroids were already extracted by dichloromethane in the former step of separation. In all *T. loureiri* leaf extracts, there were no orange-red precipitation with Dragendorff's reagent, indicating the absence of alkaloids and nitrogenous compounds.

Table 1. Phytochemical screening of *T. loureiri* extracts.

Phytochemical constituents	Test reagents	Results			
		TTL1	TTL2	TTL3	TTL4
Flavonoids	Mg/HCl	+ red	-	-	+ red
Tannins	Ferric chloride test	+ dark blue	-	-	+ dark blue
	1% gelatin test	+ precipitation	-	-	+ precipitation
Steroids/Triterpenoids	Salkowski's test	++ reddish brown	-	++ reddish brown	++ reddish brown
Alkaloids	Dragendorff's reagent	-	-	-	-

(+) sign denotes the presence of corresponding phytochemical constituents

(-) sign denotes the absence of corresponding phytochemical constituents

According to the polar extracts (TTL1 & TTL4), the major components should be flavonoids, phenolic compounds and steroids. In contrast, the components of the less polar extract (TTL3) was steroids. These were correlated to a review of Lima et al. (2014) which reported that the main constituents found in *Tetracera* species were flavonoids and triterpenoids[21]. In addition, twelve isolated compounds from *T. loureiri* stems have been recently reported. The components were also in the groups

which we estimated including flavonoids, triterpenoids and lignans[22].

3.3. Inhibition of α-glucosidase enzyme activity

α-Glucosidase enzyme has been used as a target for diabetes treatment. This enzyme is required for digestion of carbohydrate to monosaccharide prior to be absorbed into the small intestine[3,5]. The inhibition of this enzyme function therefore interferes the monosaccharide absorption leading to reduce the postprandial blood sugar. Several natural product-isolated compounds have been reported to exhibit potent α-glucosidase inhibitory activity, for example, the fruit extract of *Terminalia chebula*[23], the aqueous extract of *Morinda lucida* leaves[24] and other several natural resources[25]. Additionally, the α-glucosidase inhibitors have been found in *Tetracera* species[26,27]. In this study, the α-glucosidase inhibitory activity of *T.loureiri* leaf extracts was investigated. The enzyme activity was determined by measuring the absorption of the reaction mixture at 400 nm.

Screening of α-glucosidase enzyme inhibitory activity was performed at concentration of 50 µg/mL of each plant extract. As the result shown in Figure 1, TTL1 displayed attractive inhibition activity as 99.8%. The activity of three consequence fractions extracted from TTL1 (TTL2-TTL4) was also investigated. The dichloromethane (TTL3)- and the aqueous-methanol (TTL4)-extracts showed the high potency with percent inhibition as 96.0% and 99.3%, respectively, whereas the hexanes extract showed lower as 11.8%.

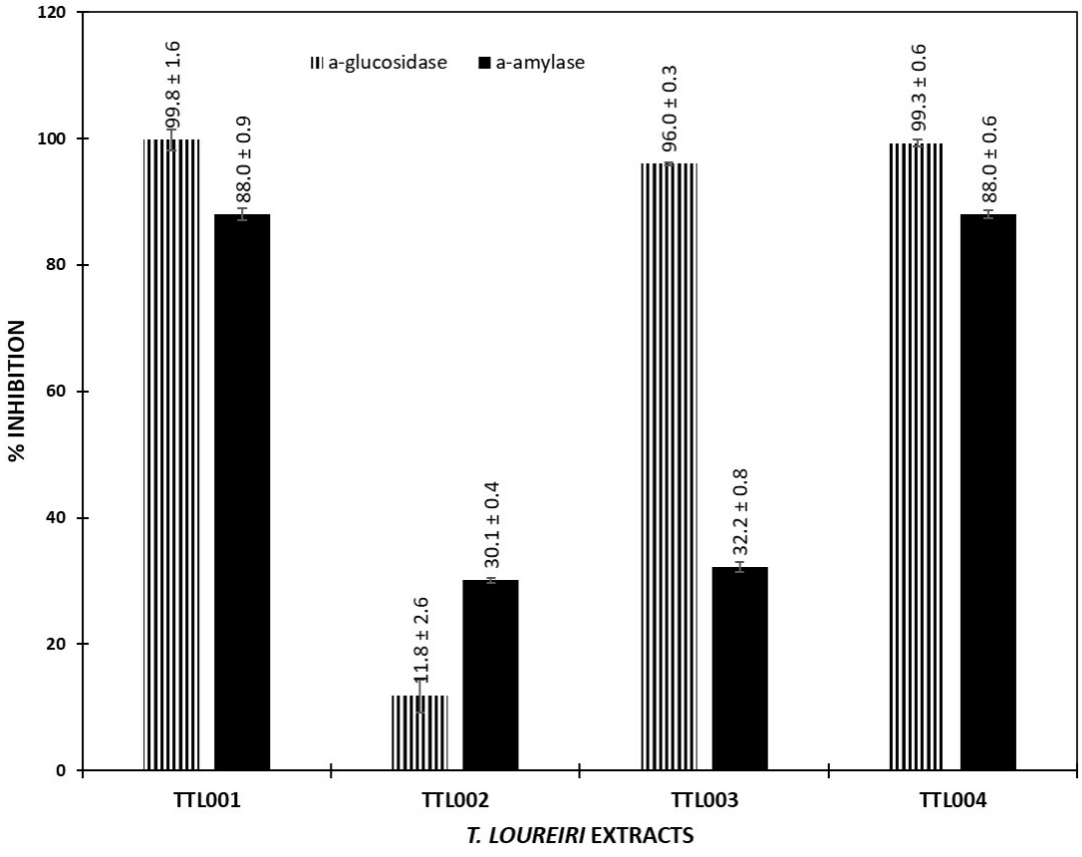

Figure 1. Percent inhibition of *T.loureiri* leaf extracts against α-glucosidase and α-amylase enzyme activities (at concentration 50 µg/mL).

The half-maximal inhibitory concentration (IC_{50}) of each highly potent extract was further determined from the relationship plot of percent inhibition vs. concentration as shown in Figure 2. The plots revealed that all extracts exhibited the α-glucosidase inhibition in dose-dependent manner. The percent inhibition of TTL1 and TTL4 have reached to more than 80% at the concentration

lower than 5 µg/mL. The IC_{50} values of tested extracts were concluded as in Table 2. The high polar extracts, TTL1 and TTL4 showed the remarkably potency at IC_{50} values as 1.8 and 1.1 µg/mL, respectively. TTL3, the moderate polar extract displayed the lower activity at IC_{50} as 10.5 µg/mL. The potency of each extract might be related to the chemical constituents. The major components of TTL1 and TTL4 were included as flavonoids and phenolic compounds which might acting as the major active constituents of both extracts. These were supported by data collected by Yin et al. (2014). As in that report, flavonoids and phenolic compounds were the major groups of naturally isolated substances which displayed high potency against α-glucosidase function[28]. The inhibition of α-glucosidase activity of *Tetracera* sp. have been previously investigated. The aqueous methanol extracts of *Tetracera scandens* leaves were reported to exhibit the IC_{50} in a range of 19.54-95.3 µg/mL[26]. The isolated flavonoids from *Tetracera indica* have been found the α-glucosidase inhibition with the IC_{50} values as 61.86-133.57 µM[27].

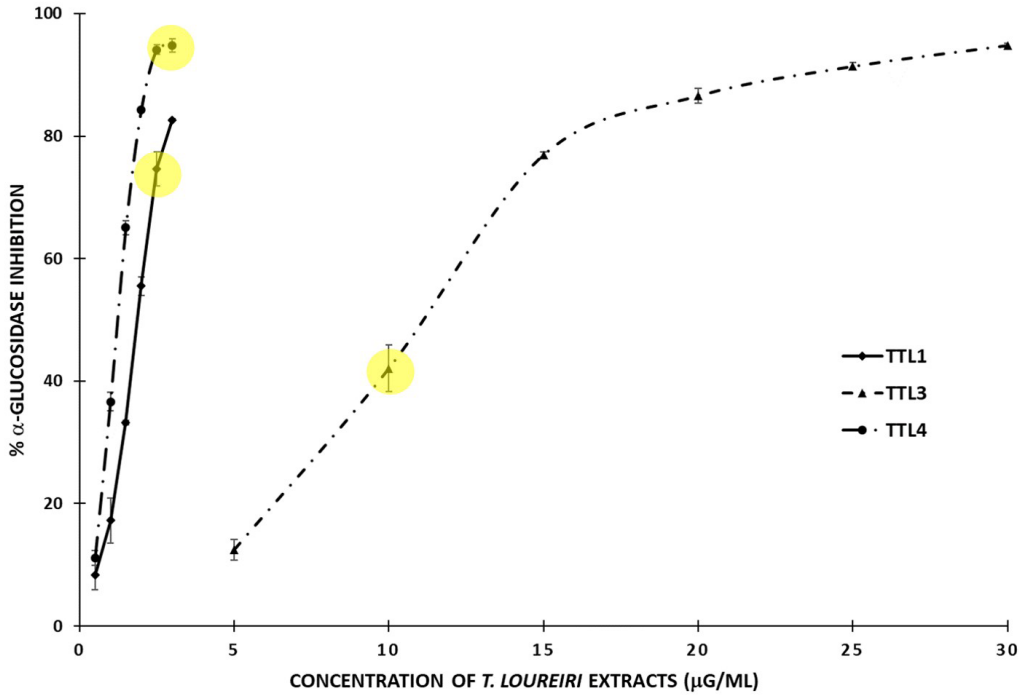

Figure 2. α-Glucosidase inhibitory effects of *T.loureiri* leaf extracts.

Table 2. α-Glucosidase and α-amylase inhibitory activities of *T. loureiri* extracts expressed in IC_{50}.

Plant extract	Enzyme inhibitory activity (IC_{50}, µg/mL)	
	α-Glucosidase	α-Amylase
TTL1	1.8	40.8
TTL2	n/a	n/a
TTL3	10.5	n/a
TTL4	1.1	13.4
Acarbose	129.6	4.5

n/a: not available

In addition, the prominent activity of flavonoids led to be studied about *in vitro* and *in silico* structure-activity relationship between flavonoids and α-glucosidase inhibition[29]. Although TTL3 showed negative results of flavonoids and phenolic testing, the enzyme inhibition activity was still presented. The activity of TTL3 might be relied on the steroid components which have also been reported to display the α-glucosidase inhibitory activity[30]. As all results, however, all three plant extracts showed the inhibition activity greater than those of a positive control, acarbose, which expressed the IC_{50} at 129.6 µg/mL.

3.4. Inhibition of α-amylase enzyme activity

The α-amylase is one of targets for interruption the carbohydrate breaking down to the absorbable monosaccharides, therefore reduces the postprandial hyperglycemia[4]. In this study, the α-amylase inhibitory activity of all *T. loureiri* extracts were investigated concurrently to α-glucosidase inhibition activity. The enzyme inhibition assay based on determination of reducing sugar produced from the reaction of α-amylase and substrate (starch). The reducing sugar was derivatized to DNS reagent and then investigated the absorption of DNS products at 540 nm[18]. Screening of α-amylase inhibition activity was determined at 50 µg/mL of *T. loureiri* leaf extracts. The high polarity fractions (TTL1 and TTL4) exhibited the percent inhibition to α-amylase activity more than 80% whereas TTL2 and TTL3 showed lower activity as 30.1% and 32.2%, respectively (Figure 1). The IC_{50} values of the active fractions were further determined from the relationship plot of percent inhibition vs. concentration as shown in Figure 3. The IC_{50} values of TTL1 and TTL4 were compared to the value of the positive control, acarbose (IC_{50} values 40.8, 13.4 and 4.5 µg/mL, respectively). These results supported the potential of TTL4 containing the interesting active compounds which appropriate for antidiabetic drug development. Depending on phytochemical screening, the possible active constituents of TTL4 might be flavonoids, phenolic compounds and steroids. There have been reported that flavonoids and phenolic compounds have the inhibitory activity of α-glucosidase that is more outstanding than other groups of substances[30-32]. Similar to α-glucosidase inhibitory activity, the structure-activity relationship of plant flavonoids and α-amylase has also been described[33].

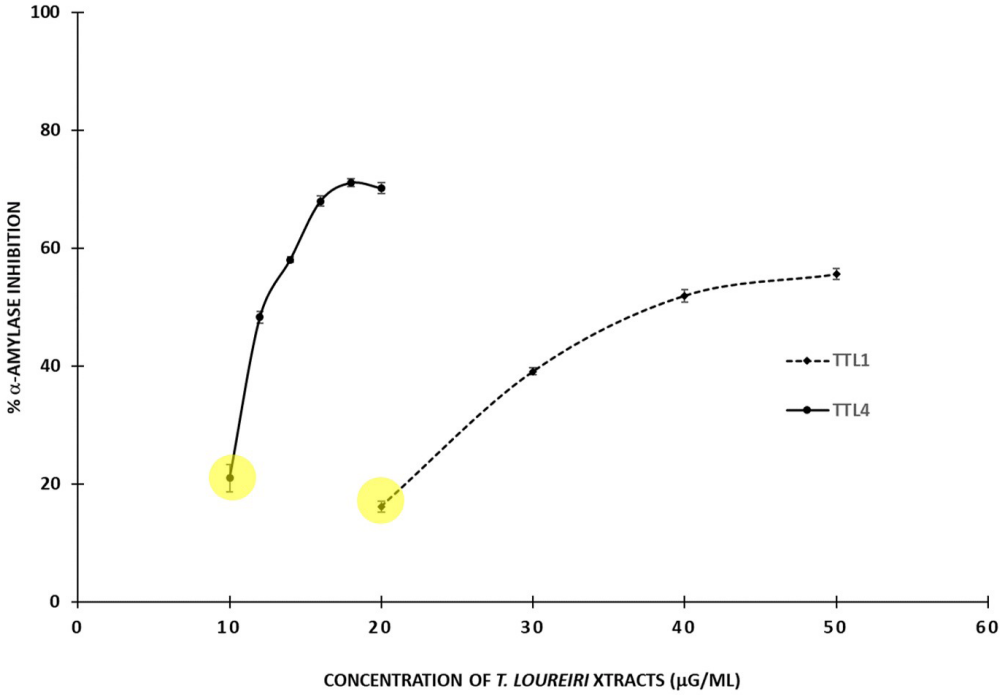

Figure 3. α-Amylase inhibitory effects of *T.loureiri* leaf extracts.

3.5. Antioxidative-related assays

Several methodologies have been proposed to assess the antioxidative activity of medicinal plants. The selection of evaluation method was chosen based on types of antioxidant of plant extract. Especially phenolic compounds have been found to act as free radical scavengers, hydrogen donors and metal chelators. In this study, the antioxidative activity of *T. loureiri* extracts was observed through two different mechanism assays including DPPH scavenging assay and Ferric Reducing Antioxidant Power (FRAP) testing. The DPPH scavenging assay was used to determine the ability of antioxidant for radical scavenging via the colorimetric chemical reaction[19]. The FRAP assay was conducted to determine the reducing capacity of antioxidant of plant extract through a redox reaction of ferric ion[20].

The DPPH scavenging activity determination of the extract was investigated over the reaction of the active substances to a stable free radical DPPH. The antioxidative activity was measured by detection of UV absorption at 510 nm. The absorption depended on the depletion of DPPH radicals presented as purple color solution. The antioxidant ability of the extracts was expressed as IC_{50} as shown in Table 3. The polar extracts, TTL1 and TTL4, displayed the potent antioxidant activity (IC_{50} values 30.60±3.86 and 10.61±2.32 μg/mL, respectively) whereas the remaining revealed the low activity. Especially TTL3 has almost no DPPH scavenging activity with IC_{50} more than 1,000 μg/mL. The results of the assay was verified by the positive control, quercetin, which showed the IC_{50} as 5.42±0.36 μg/mL.

Table 3. Antioxidative activities of *T.loureiri* extracts.

Plant extract	DPPH assay (IC_{50}, μg/ml)	FRAP assay	
		IC_{50} (μg/ml)	FRAP value (μM TE/g of extract)
TTL1	30.60±3.86[b]	20.93±0.97[a]	218.16 ± 2.31
TTL2	42.08±5.53[b]	92.59±0.65[b]	73.60 ± 1.53
TTL3	1,040.99±150.91[c]	3,152.06±2.11[c]	29.08 ± 1.27
TTL4	10.61±2.32[a]	12.45±2.11[a]	286.69 ± 36.73
Quercetin hydrate	5.42±0.36[a]	n/a	n/a
Trolox	n/a	27.67±1.24a	

*Different characters in the column mean there are significant differences (P<0.05) (n/a: not available)

The FRAP assay was also used to evaluate the antioxidative activity of *T. loureiri* extracts. The antioxidant of plant extract can reduce Fe^{3+} to be Fe^{2+}. The reaction was observed through the present of navy blue color of Fe^{2+}-TPTZ complex. The color change of the reaction was detected using microplate reader (593 nm). Similar to the results of DPPH assay, the polar part of *T. loureiri* extract, TTL1 (IC_{50} 20.93±0.97 μg/ml) and TTL4 (IC_{50} 12.45±2.11 μg/ml), exhibited the IC_{50} values as potent as a well-known antioxidant positive control, Trolox (IC_{50} 27.67±1.24 μg/ml) as shown in Table 3. The non-polar extracts also showed lower activity. TTL2 slightly inhibited the ferric reduction at IC_{50} as 92.59±0.65 μg/mL and no inhibition effects from TTL3 (IC_{50} > 3,000 μg/mL). The antioxidant activity of FRAP assay was also expressed as micromolar (μM) Trolox equivalent per gram extract (μM TE/g of extract). TTL4 expressed the greatest antioxidant power which indicated that each gram of plant extract was equivalent to 286.69 ± 36.73 μM TE/g of extract.

The effects of all *T. loureiri* leaf extracts from both DPPH and FRAP methods were corresponded to each other. TTL4 have the highest potency while TTL3 showed the lowest potency. The antioxidative activity of *T. loureiri* leaves has never been reported. Only the activity of *T. loureiri* stems have previously been studied by Kukongviriyapan et al. (2003)[12]. The ethanol extract

exhibited strong radical scavenging activity with an EC_{50} of 3.3±0.7 μg/mL, as potent as positive control (ascorbic acid). The FRAP assay indicated that each milligram of the ethanolic extract was equivalent to 1.38±0.24 μmole of ascorbic acid or 2.72±0.41 μmole of Fe^{2+} ion. The prominent antioxidative activities of both of our work and of previous literature indicated that the high polarity extract of *T. loureiri* was the attractive part for development of antidiabetic agent together with antioxidant activity.

4. CONCLUSIONS

The inhibitory activity of *T. loureiri* leaf polar extracts against the α-glucosidase expressed high potency than the result of the positive inhibitor, acarbose. Likewise, the α-amylase inhibitory activity of the polar extracts showed strong activity. These antidiabetic enzyme inhibitor fractions additionally displayed antioxidative activities of both DPPH scavenging and FRAP assay. The phytochemical screening of the extracts indicated that the possible active constituents could be flavonoids and phenolic compounds. These promising results of both anti-diabetic- and antioxidative activities screening are attracted to continue further research on *T. loureiri* for antidiabetic drugs discovery and development.

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Conflict of interest (If any)

The authors declare no conflict of interest.

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