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Chemical compositions, antioxidant, and anti-hyperglycemic activities of the extract fractions of *Terminalia bellirica* trunk-bark in streptozotocin-induced diabetic rats

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Abstract

The research aimed to evaluate the antioxidant and anti-hyperglycemic activities of different fractions of extract from the trunk bark of *Terminalia bellirica* (*T. bellirica*), a plant commonly used in traditional Asian remedies. The antioxidant activity was determined using DPPH radical-scavenging activity. The in vitro anti-hyperglycemic activity was evaluated via the inhibitory activities against α-glucosidase and α-amylase, while the measurement of fasting blood glucose reduction capacity in treated diabetic rats was used for the *in vivo* anti-hyperglycemic test. The results indicated that the increase in polarity of the extraction solvent led to higher polyphenol content and DPPH radical scavenging of the extracts. The ethyl acetate fraction (EAF) and water fraction (WF) of *T. bellirica* exhibited significantly higher α-amylase inhibitory activity (IC_{50} values of 0.118 ± 0.001 and 0.404 ± 0.001 mg/mL, respectively) than acarbose (IC_{50} = 0.492 mg/mL), but the α-glucosidase inhibitory activity of all fractions was lower than that of acarbose. In the *in vivo* test, all fractions significantly lowered fasting blood glucose levels in streptozotocin-induced diabetic rats at a dose of 200 mg/kg body weight without body weight loss, while no effect was observed with acarbose treatment. In addition, the UPLC-QTOF-MS analysis showed that the EAF contains phenolic acids, flavonoids, triterpenes, and their derivative compounds. Thus, the trunk-bark of *T. bellirica* is recommended as a potent source of natural compounds having antioxidant and anti-hypoglycemic activities.

Introduction

Terminalia bellirica is a large tropical tree genus in the *Combretaceae* family, which comprises approximately 300 species. Several *Terminalia*

species are widely used in traditional remedies in Asian countries, including Vietnam, to treat diseases that include dementia, constipation, and diabetes (Lin *et al.* 2000). The phytochemical constituents of 39 important medicinal and edible *Terminalia* species are terpenes, tannins, flavonoids, lignans, and simple phenols, as reviewed in a study by Zhang *et al.* (2019). The pharmacological studies of *Terminalia* species have indicated that they possess various beneficial biological effects such as liver and kidney protection, antibacterial, anti-inflammatory, and anticancer activities, positive effects on immune regulation, cardiovascular disease, and diabetes, and acceleration of wound healing (Zhang *et al.* 2019). Fruits of *Terminalia bellirica* and *Terminalia chebula* together with *Phyllanthus emblica* (Euphorbiaceae) used in Triphala, a Tibetan herbal remedy, showed remarkable efficiency in the treatment of cancers as well as antifungal, antimicrobial, antimalarial, antioxidant activities, as reviewed by Peterson *et al.* (2017). Our previous studies found that extracts of *T. nigrovenulosa* collected in Vietnam exhibit relatively high antioxidant activities, which are higher than those of BHT for DPPH radical scavenging and protecting oil from oxidation (Nguyen and Eun 2011b; Nguyen *et al.* 2015). In addition, *T. nigrovenulosa* extract also showed antimicrobial activity and anticancer activity (Nguyen and Eun 2011a), and the main bioactive compounds of the extract were identified as gallic acid, ethyl gallate, catechin, and luteolin (Nguyen *et al.* 2016b).

Fractionation of crude extracts by different solvents could concentrate the bioactive components in different fractions due to the compatibility of the polarity between individual compounds and each solvent. Previous findings indicated that the water fraction was the most effective for dissolving compounds from *T. algeriensis*, among others (Boulekbache-Makhlouf and Madani 2014). However, in another study, the highest antioxidant activity was found in the ethyl acetate fraction of *Combretum erythrophyllum* (Burch.) (Mtunzi *et al.* 2017). Whereas a report by Emran *et al.* (2015) showed that the chloroform fraction of five Bangladeshi plants expressed the highest clot lysis activity compared to the others. Nguyen *et al.* (2021) presented that among 5 extract fractions of trunk-bark of *T. alata*, the strongest antioxidant and hypoglycemic activities in diabetic rat models were found in the ethyl acetate and water fractions. In another study, the methanol extract from trunk-bark of *T. bellirica* exhibited significant antioxidant activity and hypoglycemic effect in a streptozocin-induced diabetic rat model (Nguyen *et al.* 2016a).

The previous findings of the variation in the phytochemical composition and biological activities in different fractions of a plant extract suggest that the fractionation of crude extract from *T. bellirica* is necessary for the isolation of its bioactive compounds and the determination of the fraction having the highest desired bioactivities. Thus, this research aimed to investigate the antioxidant and anti-hyperglycemic activities of different fractions of *T. bellirica* extract, including hexane, chloroform, ethyl acetate, n-butanol, and water. The variation in total phenolic content of the fractions was evaluated and the major phenolic components in the fractions were identified by ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS).

Experimental

Chemicals

2,2-diphenyl-2-picrylhydrazyl hydrate, gallic acid, Folin-Ciocalteu, potassium ferricyanide, sodium nitrite, aluminium chloride, streptozotocin, pancreatic α-amylase, rat intestinal acetone powder, p-nitrophenyl-α-D-glucopyranoside, and dinitrosalicylic (DNS) acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemicals and reagents reached the analytical grade.

Plant extract preparation

The collection of the trunk bark of *T. bellirica* took place in Yok Don National Park, Dak Lak Province, Vietnam. After being cut out of the tree, the barks were dried at ambient temperature with active ventilation to obtain a moisture content of less than 12 % of the drying basic, ground, and then stored at -30 ^{o}C in PE bags or directly used.

The powdered trunk bark underwent a triple extraction process using methanol, following the method outlined by Nguyen et al. (2016a). Subsequently, fractionation of the resulting crude extract was conducted according to the procedure detailed in Nguyen *et al.* (2021). Initially, the trunk bark was macerated in methanol (at a ratio of 1 : 10 w/v) and shaken at room temperature for 24 h. The resulting extracts were then filtered through No. 1 filter paper. In combination with these extracts, the solution was evaporated at 60 °C under vacuum using an IKA rotary evaporator to yield the crude extract, which was then dissolved in distilled water at a ratio of 1 : 5 w/v for fractionation. The aqueous solution underwent further fractionation with various solvents, as outlined by Nguyen *et al.* (2021). Specifically, partitioning with hexane yielded the hexane fraction (HF), while sequential partitioning of the aqueous fraction with chloroform, ethyl acetate, and n-butanol produced the chloroform (CF), ethyl acetate (EAF), and n-butanol (BF) fractions, respectively. These fractions were filtered and dried through evaporation under vacuum. Finally, the remaining aqueous fraction (WF) was filtered and dried using a freeze dryer.

Animals

For in vivo anti-hyperglycemic experiments, 5-week-old Wistar albino rats weighing between 20 and 25 g were utilized. These rats were procured from the Institute of Vaccines and Medical Biologicals (IVAC) in Nha Trang City, Vietnam. They were housed in plastic cages in a room with a 12 h light/12 h dark cycle at a temperature of 25 ± 2 °C. Prior to testing, the rats were provided with a standard rodent diet and access to water ad libitum. All experimental procedures were conducted in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at the Tay Nguyen Institute of Hygiene and Epidemiology in Dak Lak, Vietnam.

Total phenolic content and DPPH radical scavenging activity determination

The total phenolic content of the extracts was assessed employing the Folin–Ciocalteu method, with minor adjustments as described by Nguyen *et al.* (2016a).
The DPPH radical-scavenging activity was assessed following the protocols outlined by Nguyen *et al.* (2016a).

Determination of rat intestinal α-glucosidase inhibitory activity

The inhibition of rat intestinal α-glucosidase activity was conducted following the procedure established by Kwon *et al.* (2006), with minor adjustments as specified by Nguyen *et al.* (2016a). Acarbose served as the positive control in this assay.

Determination of porcine pancreatic α-amylase inhibitory activity

The inhibition of porcine pancreatic α-amylase activity was conducted in accordance with the protocol outlined by Kwon et al. (2006), with minor modifications as detailed by Nguyen *et al.* (2016a). Acarbose was employed as the positive control in this study.

Preparation of streptozotocin-induced diabetic rats

Male Wistar Albino rats were induced by ice-cold aqueous streptozotocin (120 $mg.kg^{-1}$ body weight) via intraperitoneal administration, as presented by Nguyen *et al.* (2016a). In summary, the rats were divided randomly into two groups (3 rats/group): a normal group and a diabetic group. The rats in the normal group were fed a standard chow diet, while those in the diabetic group were placed on a high-fat diet. Both groups adhered to their respective diets for a period of six weeks. Rats in the diabetic group were induced with streptozotocin, receiving a single intraperitoneal injection of ice-cold aqueous streptozotocin at a dose of 120 $mg.kg^{-1}$. After three days, blood glucose levels were measured using a Bionime GM110 blood glucose meter (Bionime, Switzerland). Rats exhibiting marked hyperglycemia (fasting blood glucose level > 10 $mmol.mL^{-1}$) were selected for the study. Throughout the experiment, all animals had unrestricted access to tap water and pellet diets and were housed in plastic cages at room temperature.

Evaluation the effects of extract fractions on streptozotocin-induced diabetic rats

The experiments for single and multi-dose experiments followed the method described by Nguyen *et al.* (2016a). The blood glucose meter (Bionime GM110, Bionime, Swiss) was used for the measurement of fasting glucose levels.

Analysis of secondary compounds by UPLC-QTOF-MS

The extract was analyzed by an ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) analytical system (SCIEX-X500R QTOF, USA), The instrument consisted of an ExionLC^{TM} system coupled with a X500R QTOF mass spectrometer with Turbo V^{TM} source and Electrospray Ionization (ESI) was used. Each sample was injected in six replicates at a volume of 2 µL. Blank (100 % MeOH) was run at the beginning of the sample sequence for the elimination of background features. Compound separation was performed using a Hypersil GOLD (150 × 2.1 mm; 3µm; Thermo Scientific, USA) at 25 ºC. The mobile phases were 0.1 % formic acid in water (A) and 0.1 % formic acid in ACN (B) with the following gradients: 98 % A (0 – 1 min), 98 % - 2 % A (1 – 20 min) and finally 2 % A (20 – 25 min). The flow rate was 0.4 mL.min^{-1}. The MS experiment was performed with Turbo V^{TM} source and Electrospray Ionization (ESI) in negative polarity under the following conditions: TOF-MS survey (70 – 2,000 Da for 250 msec) using collision energy (CE) of -20 V with collision energy spread (CES) of 10 V, and respective Declustering potential and Declustering potential spread were -80 V and 0 V. TOF-MS/MS scans (50 – 1,500 Da for 100 msec). The MS/MS fragmentation was achieved using collision energy (CE) of -35 V with collision energy spread (CES) of 10 V. Declustering potential and Declustering potential spread were -80 V and 0 V, respectively.

Data analysis

All experiments were carried out in triplicates, and the results were shown as the mean values ± standard deviations. The significant statistic for each experiment was determined using the analysis of variance test (ANOVA) and the LSD (Least Significant Difference). A post-test was used for the comparison of the mean values. Differences were considered significant at $P < 0.05$.

Results

Total polyphenol contents and DPPH inhibitory activity of T. bellirica *fractions*

The highest total polyphenol content was found in the ethyl acetate fraction (EAF), followed by the *n*-butanol (BUF) and water (WF) fractions (522.58 mg GAE/g, 456.03 mg GAE/g, and 383.51 mg GAE/g, respectively, Table 1). The lowest content of total polyphenol was observed in the hexane fraction (HF), followed by the chloroform fraction (ChF). The IC_{50} values of DPPH radical scavenging activity indicated that the DPPH radical scavenging activity of all extract fractions was lower than that of the positive control (ascorbic acid). As shown in Table 1, the fractions containing higher total phenolic content presented stronger free radical scavenging activity, suggesting that phenolics are the main components responsible for the DPPH radical scavenging activity of the fractions.

Table 1. Total polyphenol contents and DPPH radical scavenging activity of the fractions from *T. bellirica.*

Samples/control	Total phenolics content [mg GAE/g]	DPPH radical scavenging activity [IC_{50}, mg/mL]
HF	24.13 ± 0.08^{e}	11.19 ± 0.20^{a}
ChF	95.60 ± 0.21^{d}	5.58 ± 0.42^{c}
EAF	522.58 ± 2.56^{a}	0.42 ± 0.01^{d}
BF	456.03 ± 2.38^{b}	0.53 ± 0.01^{c}
WF	383.51 ± 3.47^{c}	0.94 ± 0.01^{b}
Ascorbic acid	-	0.240 ± 0.010^{e}

Different letters indicate significant differences among the values within a column at $P < 0.05$. (-) no detection. HF, CF, EAF, BF, and WF are hexane, chloroform, ethyl acetate, n-butanol, and water extract fractions, respectively.

Moreover, the study with the crude extract of *T. bellirica* (Nguyen *et al.* 2016a) also expressed that active compounds of different polarities could be present in this plant extract, mainly concentrated in EAF, BuF, and WF which are in the range of low to high polarity.

Alpha-amylase and alpha-glucosidase inhibitory activities of fractions

Significant differences in inhibitory activities against the enzymes (α-amylase and α-glucosidase) of fractions were observed via IC_{50} values (Table 2). EAF possessed the highest inhibitory activity against α-amylase (IC_{50} = 0.118 mg.mL^{-1}) which was much higher than that of the standard acarbose, an anti-diabetic drug (IC_{50} = 0.492 mg.mL^{-1}). The second highest inhibition against α-amylase was followed by WF with an IC_{50} of 0.404 mg.mL^{-1}, and the lowest effect was found in the treatment with ChF. The HF and ChF did not express α-glucosidase inhibitory activity at the investigated concentration. EAF also possessed the highest inhibitory activity against α-glucosidase (IC_{50} = 0.380 mg.mL^{-1}), followed by BuF (IC_{50} = 1.896 mg.mL^{-1}) which was much higher than the IC_{50} of acarbose (0.154 mg.mL^{-1}). In comparison with the enzyme inhibitory activity of the crude extract of *T. bellirica* in our previous study (Nguyen *et al.* 2016a), the fractionation in this study could successfully concentrate the compounds of the crude extract by using appropriate solvents, especially in the ethyl acetate fraction (EAF).

Table 2. The α-amylase and α-glucosidase inhibitory activities of *T. bellirica* fractions.

Sample/Controls	α-glucosidase inhibition IC_{50} [mg.mL^{-1}]	α-amylase inhibition IC_{50} [mg.mL^{-1}]
HF	-	3.862 ± 0.126^{b}
CF	-	6.965 ± 0.051^{a}
EAF	0.380 ± 0.007^{c}	0.118 ± 0.001^{e}
BF	1.896 ± 0.003^{b}	0.844 ± 0.010^{c}
WF	2.450 ± 0.004^{a}	0.404 ± 0.001^{d}
Acarbose	0.154 ± 0.020^{d}	0.492 ± 0.020^{d}

Different letters indicate significant differences among the values within a column at $P < 0.05$. (-) no detection. HF, CF, EAF, BF, and WF are hexane, chloroform, ethyl acetate, n-butanol, and water extract fractions, respectively.

Acute toxicity and effect on glucose level in normal rats

The normal rats were daily fed with the fractions of *T. bellirica* by oral intubation at a dose of 100, 200, and 300 mg.kg^{-1} body weight and observed for eight days. The results in Table 3 indicated that the administration of the fractions from the *T. bellirica* extract at oral doses up to 300 mg.kg^{-1} body weight did not cause any significant change in the glucose level of the normal rats.

Table 3. Blood glucose level of normal rats administrated with fractions of *T. bellirica* extract.

No.	Samples/ Controls	Oral dose [mg.kg^{-1} body weight]	Blood glucose concentration [mmol.mL^{-1}]		
			Day 0	Day 4th	Day 8th
1	EAF	100	7.33 ± 0.46	6.83 ± 0.47	6.80 ± 0.46
		200	7.33± 0.10	7.07 ± 0.36	6.80 ± 0.45
		300	8.93 ± 0.49	8.60 ± 0.36	8.17 ± 0.45
2	BF	100	8.57 ± 0.68	7.90 ± 0.78	7.27 ± 0.57
		200	8.73 ± 0.55	8.00 ± 0.66	8.00 ± 0.36
		300	8.23 ± 0.47	7.33 ± 1.16	7.63 ± 0.40
3	WF	100	7.73 ± 0.97	7.27 ± 0.86	7.23 ± 0.59
		200	8.90 ± 0.53	8.47 ± 0.23	8.40 ± 0.30
		300	8.83 ± 0.45	8.40 ± 0.61	7.97 ± 0.46
4	Saline		9.00 ± 1.81	8.50 ± 0.35	7.73 ± 0.66

HF, CF, EAF, BF, and WF are hexane, chloroform, ethyl acetate, n-butanol, and water extract fractions, respectively.

Regarding the evaluation of acute toxicity, neither mortality nor any abnormal signs of the status of the treated rats were observed during the 8-day period of the experiment. Therefore, the dose of 200 mg.kg^{-1} body weight was used as the safe dose for investigation of anti-hypoglycemic activity of all fractions.

Effect of the fractions on fasting blood glucose levels in diabetic rats (single dose)

Since the extraction yields of ChF and HF expressed relatively low inhibitory activities against the starch hydrolyzing enzymes, the *in vivo* experiments in rat models were only carried out using three fractions including EAF, BuF and WF. Table 4 presents the effects of the treatments with *T. bellirica* fractions, acarbose (positive control), and saline (negative control), on blood glucose levels in streptozotocin-induced diabetic rats. The fasting blood glucose levels of streptozotocin-induced diabetic rat models used in this study were around 12 to 14 mmol.mL^{-1}. In the single dose evaluation, the acarbose treatment led to the maximum effect within 2 – 4 h after the administration, and then the blood glucose level of rats slightly increased after 6 – 8 h, whereas the EAF and WF had a significant effect within 4 – 8 h after treatment. The results in Table 4 suggest that the blood glucose level in diabetic rats administered with the extract fractions was slowly reduced and kept constant for a longer time as compared to the acarbose treatment (positive control). In contrast, the glucose level in rats treated with saline kept constant until 6 h and significantly increased after 8 h.

Table 4. Fasting blood glucose levels in diabetic rats (single dose) treated with fractions at a dose of 200 mg.kg^{-1} body weight.

Samples/ Controls	Blood glucose concentration [mmol.L^{-1}]				
	0 h	2 h	4 h	6 h	8 h
EAF	12.60 ± 0.70^{a}	10.20 ± 1.5^{a}	6.77 ± 0.70^{b}	7.40 ± 0.62^{b}	7.27 ± 0.12^{b}
BF	13.63 ± 1.42^{a}	10.77 ± 0.73^{a}	9.57 ± 1.69^{a}	9.57 ± 1.30^{a}	9.10 ± 1.13^{a}
WF	14.00 ± 0.30^{a}	9.67 ± 1.01^{a}	6.80 ± 0.26^{b}	7.57 ± 0.83^{b}	7.67 ± 0.45^{b}
Positive control (Acarbose)	14.10 ± 1.93^{a}	7.10 ± 0.95^{b}	8.17 ± 1.52^{b}	9.10 ± 0.62^{b}	11.23 ± 2.05^{a}
Negative control (saline)	13.37 ± 2.01^{b}	12.33 ± 1.90^{b}	13.57 ± 2.10^{b}	13.40 ± 1.75^{b}	14.50 ± 0.96^{a}

Different letters indicate significant differences among the values within a row at $P < 0.05$. EAF, BF, and WF are hexane, chloroform, ethyl acetate, n-butanol and water extract fractions, respectively.

Among the extract fractions, WF and EAF caused the highest reduction of glucose level in streptozotocin-induced diabetic rats (Table 4). The respective percentage of glucose level decreased after 2, 4, 6, and 8 h in rats treated with WF was 30.95 %, 51.43 %, 45.95 %, and 45.23 % and that of rats treated EAF was 19.05 %, 46.29 %, 41.27 %, and 42.32 % compared to the glucose level at 0 h after treatment. The highest decrease in glucose level in rats (46.29 % to 51.43 %) was found in rats treated with EAF and WF for 4 h, and the glucose level reached no significant change until 8 h. The results indicated that the fractions of *T. bellirica* extract can significantly reduce the blood glucose level in diabetic rat models.

Effect of multiple doses of fractions on fasting blood glucose levels in diabetic rats

The levels of glucose in diabetic rats treated with different *T. bellirica* fractions and controls are shown in Table 5. Generally, daily oral treatment of the fractions and controls at a safe dose could induce a decrease in the glucose level of the diabetic rats. The reduction in postprandial blood glucose level in diabetic rats of the fractions and acarbose was obviously observed after 4 days of the treatments and then remained unchanged until the 12th day. Acarbose showed a higher effect on the reduction in postprandial blood glucose levels in diabetic rats than the extract fractions. Amongst the extract fractions, EAF resulted in the highest reduction in postprandial blood glucose level, which was 25.6 %, 39.3 %, and 43.1 % after the treatment for 4, 8, and 12 days, respectively. In contrast, there was a significant increase in the glucose level of diabetic rats treated with saline

(the control) during the investigated period, which was 152.8 % after 12 days compared to the initial glucose level of the rats. These results imply that both single and multiple doses of *T. bellirica* extract fractions and acarbose can cause efficient hypoglycemic activity in streptozotocin-induced diabetic rats.

Table 5. Effect of multiple doses of fractions on blood glucose levels in diabetic rats as treatment days.

Samples / controls	Concentration of blood glucose [mmol.L^{-1}]			
	Day 0	Day 4th	Day 8th	Day 12th
EAF	11.70 ± 1.50^{a}	8.80 ± 0.87^{b}	7.07 ± 0.81^{b}	6.63 ± 0.74^{b}
BF	12.83 ± 0.78^{a}	10.17 ± 2.29^{c}	7.93 ± 0.31^{b}	7.83 ± 0.25^{b}
WF	12.80 ± 0.79^{a}	9.70 ± 1.35^{b}	9.83 ± 0.06^{b}	9.23 ± 0.25^{b}
Positive control (Acarbose)	13.20 ± 3.21^{a}	10.00 ± 5.90^{a}	6.90 ± 3.75^{b}	5.93 ± 2.27^{b}
Diabetic rats (saline)	10.70 ± 0.50^{b}	10.70 ± 1.22^{b}	13.37 ± 2.42^{ab}	16.23 ± 3.19^{a}
Normal rats (saline)	7.43 ± 0.32^{a}	7.53 ± 0.46^{a}	7.10 ± 0.66^{a}	6.90 ± 0.79^{a}

Different letters indicate significant differences among the values within a row at $P < 0.05$. (-) no detection. EAF, BF, and WF are hexane, chloroform, ethyl acetate, n-butanol and water extract fractions, respectively.

In addition, different treatments also caused significant variations in the change in body weight amongst the investigated groups (Fig. 1). The acarbose-treated diabetic rats expressed the highest reduction in body weight during treatment duration (3.71 % – 24.15 %). However, the body weight of fraction-treated diabetic rats and normal rats only varied around ± 5 % compared to the initial body weight. Regarding the diabetic rats treated with saline, their body weight slightly increased after the first 4 days of treatment but then significantly reduced after 8 to 12 days. The results indicated that the *T. bellirica* extract fractions can induce a glucose reduction but do not cause significant body weight loss in diabetic rats compared to treatments with acarbose and saline.

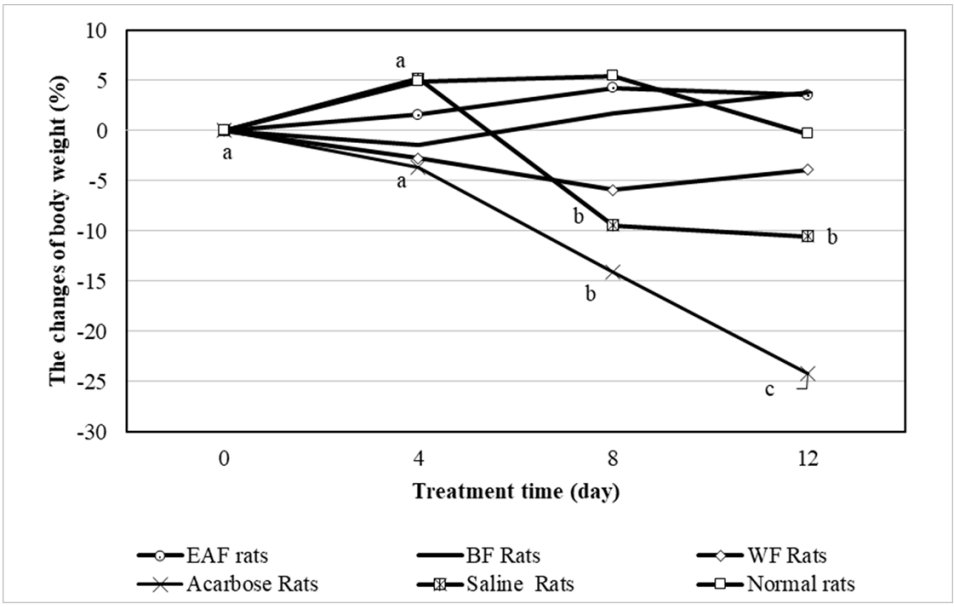


Fig. 1. Effect of fractions on the body weight of diabetic rats (multiple doses). Different letters above the line for the same fraction-treatment, saline-treatment, or acarbose-treatment indicate statistically significant differences at $P < 0.05$. EAF rats, BF Rats, WF rats Acarbose rats, and saline Rats are streptozotocin-induced diabetic rats treated with hexane, chloroform, ethyl acetate, n-butanol, water extract fractions, acarbose and saline, respectively. Normal rats are normal rats treated with saline.

Identification of secondary metabolites by UPLC-QTOF-MS

The result of the UPLC-QTOF-MS analysis of secondary compounds in the EAF of *T. bellirica* extract is presented in Table 6. The analysis

indicated that the major compounds in the EAF were phenolic acids, flavonoids, triterpenes, and their derivatives. Among the identified compounds, (+)-catechin and ellagic acid in *Terminalia bellirica* have been reported by Sobeh *et al.* (2017). However, flavanomarein, salvianolic acid B, madecassic acid, and caulophyllogenin were the first report for *Terminalia bellirica* collected in Yok Don National Park, Dak Lak Province, in this study.

Table 6. Tentative identification of secondary metabolites of EAF of *T. bellirica* extract.

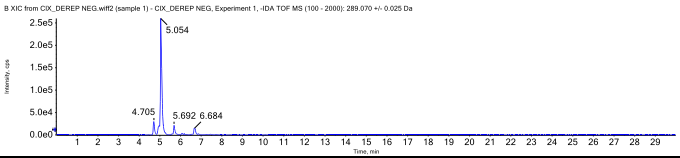
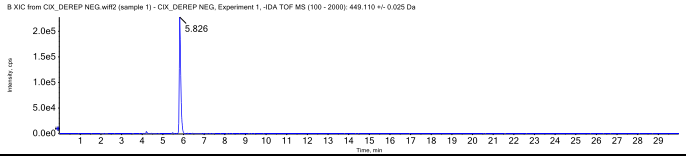
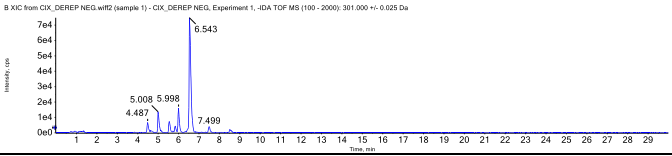
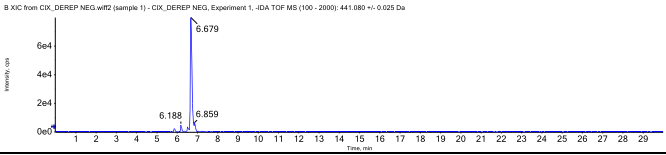
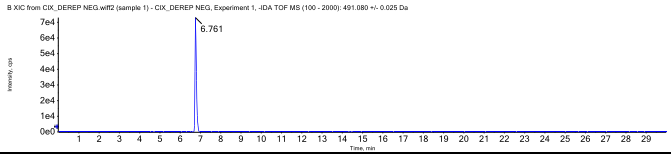
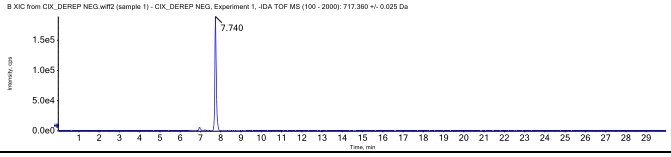
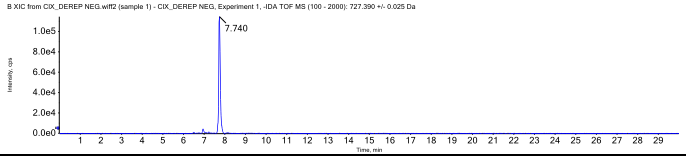
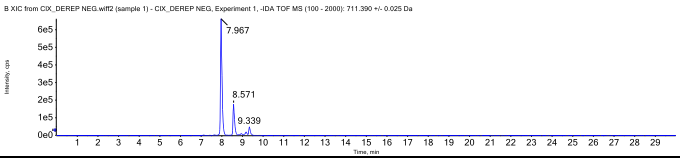
No.	Retention time	Precursor mass	Suggested compounds	XIC
1	5.05	289.0730	(+)-Catechin	
2	5.83	449.1101	Flavanomarein	
3	6.54	301.0006	Ellagic acid	
4	6.68	441.0843	(-)-Catechin gallate	
5	6.76	491.0846	Ellagic acid; 3,8-Di-Me ether, 2-*O*-β-D-glucopyranoside	
6	7.74	717.3621	Salvianolic acid B	
7	7.74	727.3912	2,3,19,23,24-Pentahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form, 28-*O*-β-D-Galactopyranosyl ester	
8	7.97	711.3960	1,3,9,22-Tetrahydroxy-12-oleanen-28-oic acid; (1α,3β,9α,22α)-form, 3-*O*-β-D-Glucopyranoside	

Table 6. Tentative identification of secondary metabolites of EAF of *T. bellirica* extract (continued).

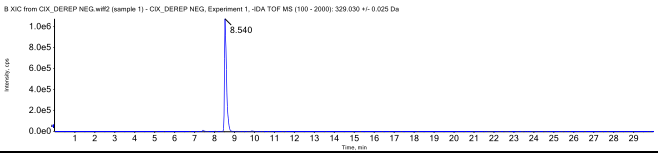
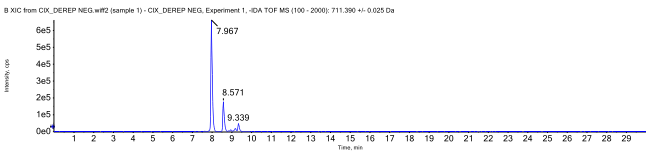
9			2,3,6,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,6β)-form, 28-*O*-β-D-Glucopyranosyl ester	
10			2,3,7,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,7α)-form, β-D-Glucopyranosyl ester	
11			2,3,19,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form, 28-β-D-Glucopyranosyl ester	
12			2,3,19,24-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form, 28-*O*-β-D-Glucopyranosyl ester	
13			2,3,23,24-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β)-form, 28-*O*-β-D-Glucopyranosyl ester	
14			2,3,23,27-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β)-form, 28-*O*-β-D-Glucopyranosyl ester	
15	8.54	329.0311	3,4'-Dimethoxy-5,7,3'-trihydroxyflavone	
16	8.57	711.3964	1,3,9,22-Tetrahydroxy-12-oleanen-28-oic acid; (1α,3β,9α,22α)-form, 3-*O*-β-D-Glucopyranoside	

Table 6. Tentative identification of secondary metabolites of EAF of *T. bellirica* extract (continued).

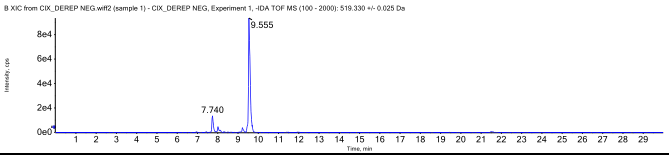
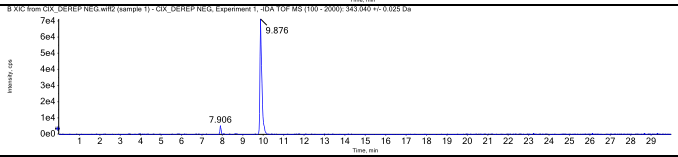
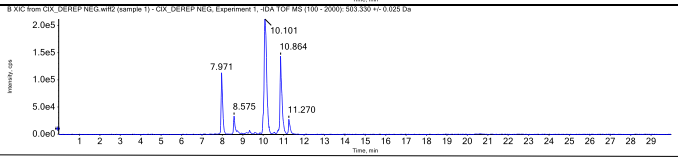
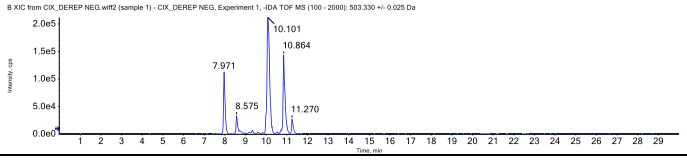
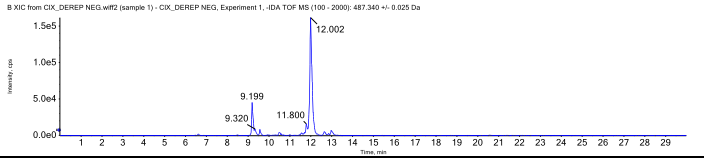
17			2,3,6,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,6β)-form, 28-*O*-β-D-Glucopyranosyl ester	
18			2,3,7,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,7α)-form, β-D-Glucopyranosyl ester	
19			2,3,19,23-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form, 28-β-D-Glucopyranosyl ester	
20			2,3,19,24-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form, 28-*O*-β-D-Glucopyranosyl ester	
21			2,3,23,24-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β)-form, 28-*O*-β-D-Glucopyranosyl ester	
22			2,3,23,27-Tetrahydroxy-12-oleanen-28-oic acid; (2α,3β)-form, 28-*O*-β-D-Glucopyranosyl ester	
23	9.55	519.3335	2,3,19,23,24-Pentahydroxy-12-oleanen-28-oic acid; (2α,3β,19α)-form	
24	9.88	343.0475	5,3'-Dihydroxy-6,7,4'-trimethoxyflavone	
25	10.10	503.3385	Madecassic acid	

Table 6. Tentative identification of secondary metabolites of EAF of *T. bellirica* extract (continued).

26	10.86	503.3386	Madecassic acid	
27	12.00	487.3442	Caulophyllogenin	

Discussion

Previous studies have indicated that active compounds with different polarities might be present in the plants and are preferable to be dissolved in different types of solvents. The findings of this study showed that the fractions of crude extract of *T. bellirica* obtained from different polarity solvents contained significantly different contents of total polyphenols. Moreover, a high correlation between total phenolic content and IC_{50} values of DPPH radical scavenging activity (correlation coefficient of -0.937 and R^2 0.877 of the linear regression equation) suggests that the phenolic group is highly responsible for the DPPH radical scavenging activity of the fractions. The results in this study are in accordance with the previous reports, which also stated the high correlations between total phenolic content and antioxidant activity of extracts from medicinal plants, grains, and vegetables (Piljac-Žegarac *et al.* 2007; Nguyen and Eun 2011b; Kunyanga *et al.* 2012; Tsun-Thai and Fai-Chu 2012).

Regarding the correlation between total polyphenol content in the fractions and the IC_{50} value of the inhibitory activities against starch hydrolyzing enzymes, relatively high R square values (α-glucosidase, R^2 = 83.79 %; α-amylase, R^2 = 74.31 %) of the linear regression equations were found in this study. In addition, the correlation coefficients (R values) between the respective IC_{50} values of α-glucosidase and α-amylase inhibitory activities and the total polyphenol content of -0.915 and -0.936, respectively, confirmed that phenolic components are the major compounds contributing to the starch hydrolyzing enzyme inhibitory activities of the fraction. Phenolic compounds have been indeed reported to possess both antioxidant activity and lowering postprandial hyperglycemia by inhibiting carbohydrate hydrolyzing enzymes in the context of diabetes (Hanamura *et al.* 2005; Kwon *et al.* 2006). Moreover, they could increase the sensitivity of insulin and stimulate glycogen synthesis and the antioxidative system (Xie *et al.* 2013; Huang *et al.* 2015; Arancibia-Radich *et al.* 2019). Previous studies also stated the ability of phenolic compounds to control glycemic and hemostatic imbalance as well as lower lipid, and attenuate oxidative and inflammatory stress in diabetic mice (Huang *et al.* 2015; Yuan-Man *et al.* 2015).

The hydrolysis of starch by pancreatic α-amylase and intestinal α-glucosidase to glucose, which is easily absorbed by the small intestine, is responsible for sudden increase in glucose levels in type 2 diabetes patients (Gray 1975). Therefore, the utilization of intestinal α-glucosidase and pancreatic α-amylase inhibitors is the most potent therapy for controlling hyperglycemia in type 2 diabetes (Krentz and Bailey 2005). Previous studies reported that the extracts of several *Terminalia* species possessed anti-hyperglycemic activity. For examples, in our previous study, Nguyen *et al.* (2016a) found that the crude methanol extract of *T. bellirica* possessed high inhibitory activities against α-amylase and α-glucosidase and anti-hyperglycemic activity. The diabetic rats treated with crude extracts of *T. bellirica* at a dose of 200 $mg.kg^{-1}$ bw induced a glucose level reduction of 45.40 % after 4-h treatment**Chyba! Nenašiel sa žiaden zdroj odkazov.**. Sabu and Kuttan (2002) reported that the methanol extracts of *T. chebula* and *T. bellirica* at a dose of 100 $mg.kg^{-1}$ bw could result in decreases of

12.7 % and 13.1 % of glucose levels in diabetic rats after 4 h of treatment, respectively.

Meanwhile, the reduction rate of blood glucose in diabetic rats treated with respective WF and EAF for 4 h at the same conditions in this study was 51.43 % and 46.29 % after. Diabetic rats treated with both single and multiple doses of *T. bellirica* extract fractions showed an efficiency in reduction of glucose levels, while no effect was observed on normal rats (Table 4). The results may be due to a homeostasis in normal rats that could control the normal regulatory mechanisms involved in carbohydrate metabolism (Vats *et al.* 2002). The blood glucose level in diabetic rats significantly reduced after the 4-day treatment with EAF and WF and after the 8-day treatment with BF and acarbose (Table 4) before remaining constant until the 12th day. The results in Table 2 and Table 4 indicated that there was a positive relationship between the α-amylase and α-glucosidase inhibitory effects and the efficiency in lowering blood glucose levels in diabetic rats of the extract fractions. Therefore, the inhibition of α-amylase and α-glucosidase leads to the inhibition of gluconeogenesis and also the reduction of the absorption of glucose from the intestine, which is considered the main factor affecting post-prandial hyperglycemia after a meal (Youn *et al.* 2004).

The major secondary compounds in the EAF, including salvianolic acid B, (+)-catechin, madecassic acid, ellagic acid, 5,3'-Dihydroxy-6,7,4'-trimethoxyflavone, 3,4'-Dimethoxy-5,7,3'-trihydroxyflavone, flavanomarein, and other triterpenes (Table 6), have been considered as candidates for antidiabetic activity. Salvianolic acid B, (+)-catechin, and madecassic acid could increase the sensitivity of insulin and stimulate glycogen synthesis and the antioxidative system (Xie *et al.* 2013; Huang *et al.* 2015; Samarghandian *et al.* 2017; Arancibia-Radich *et al.* 2019), control glycemic and hemostatic imbalance, lower lipid levels, and attenuate oxidative and inflammatory stress in diabetic mice (Huang *et al.* 2015; Yuan-Man *et al.* 2015). Moreover, ellagic acid, ellagic acid nanoscale, and salvianolic acid B improved the action on β-cells of pancreas and exhibited anti-apoptosis effects (Raoufi *et al.* 2015; Fatima *et al.* 2017; Harakeh *et al.* 2020). The anti-diabetic activity of flavanomarein was reported by its ability to inhibit the epithelial-mesenchymal transition in HK-2 cells via interacting with spleen tyrosine kinase (Nan-nan *et al.* 2020). These findings and the results of the UPLC-QTOF-MS analysis provide evidence supporting the significant contribution of the secondary compounds to the antidiabetic activity of the ethyl acetate fraction of *T. bellirica* extract.

Conclusions

The findings of the current study indicate that there is a positive correlation between total polyphenol content and DPPH radical scavenging as well as hydrolyzing starch enzyme inhibitory activities of the *T. bellirica* extract fractions. Among the fractions, EAF is the most potent candidate for inducing antioxidant and hyperglycemic activities. In addition, the UPLC-QTOF-MS analysis results showed that the major compounds found in the extract were phenolic acids, flavonoids, triterpenes, and their derivatives. Among those, flavanomarein, salvianolic acid B, madecassic acid, and caulophyllogenin were the first to be identified in *Terminalia bellirica* collected in Yok Don National Park, Dak Lak Province, Vietnam.

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Conflict of Interests

The authors declare that they have no conflict of interest.

Statement of Human and Animal Rights

All experiment procedures involving animals were conducted under the regulation of the Institutional Animal Care and Use Committee at the Tay Nguyen Institute of Hygiene and Epidemiology, Dak Lak, Vietnam.

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