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Evaluation of enzyme inhibitory and antioxidant activity of some Lamiaceae plants

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ABSTRACT: Lamiaceae is one of the most widespread families in Turkey. The aim of this study was to determine antialzheimer, antidiabetic, antioxidant and antiobesity activities of ethanol extracts of *Lamium purpureum* var. *purpureum*, *Origanum onites*, *Salvia sclarea*, *S. virgata* and *Thymus zygioides* var. *lycaonius*. Acetylcholinesterase, butyrylcholinesterase, α-amylase, α-glucosidase, and pancreatic lipase inhibitory activities were tested for the determination of the activity of these extracts. Furthermore, total antioxidant, ferric-reducing antioxidant power, metal-chelating and *N,N*-dimethyl-*p*-phenylendiamine radical scavenging assays were utilized to screen antioxidant activity. Total phenolic content of the extracts were also calculated. Among the tested extracts, *T. zygioides* var. *lycaonius* aerial part extract (85.28 ± 0.89 %) showed the highest inhibitory activity against α-glucosidase. The inhibitory activities of all extracts against α-amylase were lower than 50 %. *S. sclarea* leaf extract indicated remarkable butyrylcholinesterase inhibition (51.76 ± 1.04 %), but all of the plant extracts were inactive against acetylcholinesterase. The *Salvia* species showed the highest total antioxidant activity. *S. sclarea* flower (1.34 ± 0.08) and leaf (1.34 ± 0.08) extracts showed the highest ferric-reducing antioxidant power activities. Our findings indicated that *O. onites*, *S. sclarea*, *S. virgata*, and *T. zygioides* var. *lycaonius* extracts showed valuable inhibitory activity and emerged as the sources of possible α-glucosidase inhibitors for future studies.

KEYWORDS: Lamiaceae; anticholinesterase; antidiabetic; antiobesity; antioxidant.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder caused by impairments in insulin action or secretion. Hyperglycemia, which is constantly present in diabetes patients, causes various chronic disorders such as nephropathy, neuropathy, and retinopathy [1]. It has been indicated that the high blood sugar also causes increased free radicals and decreased antioxidant markers [2]. Free radicals and intracellular antioxidant mechanisms play essential roles in DM and pathogenesis of Alzheimer's disease (AD).

Reduction of absorption of carbohydrates is one of the approaches in the treatment of DM. Thus, the postprandial rise of blood sugar is reduced. Inhibition of α-amylase and α-glucosidase enzymes, involved in the digestion of oligosaccharides and polysaccharides, can reduce increase of glucose in the blood significantly [3]. These two enzyme systems are widely used *in vitro* antidiabetic activity screening studies.

Obesity is a metabolic disorder, which is characterized by excess fat accumulation. It is demonstrated that obesity may cause many of chronic diseases such as dementia, type 2 DM, hypertension, dyslipidemia and depression [4]. The most important target of treatment of obesity is inhibition of food digestion and absorption to the blood. Inhibition of pancreatic lipase and lipid absorption is a very effective approach to find effective drugs for the treatment of obesity. Many plants, bacteria, fungi and marine organisms have been screened to find new compounds that are inhibiting pancreatic lipase for the treatment of obesity [5].

Pancreatic lipase inhibition is the most commonly used enzymatic method in obesity researches. Triglycerides are digested to fatty acids by the action of pancreatic lipase. When pancreatic lipase is inhibited, dietary fats cannot be absorbed into blood [6]. This way, people who consume fatty foods may be prevented from gaining weight. According to the results of our pancreatic lipase inhibitory activity, it was revealed that the plants tested are not effective in obesity control due to their low capacity to inhibit pancreatic lipase.

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It is well known that cognitive dysfunction and dementia are important complications of DM and obesity that reduce quality of life [7, 8]. The sister enzymes; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are associated with the formation of dementia types, AD in particular. Therefore, cholinesterase inhibitors are currently the most used drug class in the treatment of AD. Although inhibition of both enzymes contributes to AD treatment, recent studies have reported that BChE inhibition will also lead to more effective treatment [9].

Lamiaceae is one of the most widespread families that contains 45 genera and 550 species in Turkey. It is rich in plants with several biological activities and many species of the family are utilized traditionally for various diseases [10]. The infusions of *Origanum onites* (kekik) and *Thymus zygioides* var. *lycaonicus* (kekik, kır çayı, taş kekiği) are used traditionally in treatment of DM [11-13]. It has been indicated that the species of *Salvia* have been used against dementia in Europe [14]. On the other hand, many researchers have conducted studies on the antidiabetic activities of *Salvia* and *Lamium* species to date [15, 16]. Also, literature survey clearly demonstrated that there are many studies on anticholinesterase activity of different *Origanum*, *Salvia*, and *Thymus* species [17-19].

Because of these reasons, the present study assessed the *in vitro* antioxidant, anticholinesterase, antiobesity and antidiabetic potentials of five Lamiaceae plants (*Lamium purpureum* var. *purpureum* L., *Origanum onites* L., *Salvia sclarea* L., *S. virgata* Jacq., *Thymus zygioides* Griseb. var. *lycaonicus* (Celak.) Ronniger) by using different biochemical enzyme and chemical reagent assays. Additionally, total phenolic contents of these plants were determined using the Folin Ciocalteu method.

2. RESULTS

In this study, the ethanol extracts of the aerial parts of *L. purpureum* var. *purpureum*, *O. onites, S. virgata, T. zygioides* var. *lycaonius*, and leaves and flowers of *S. sclarea* were screened against α-amylase and α-glucosidase, AChE, BChE and pancreatic lipase enzymes. Antioxidant activity of the extracts was also determined by total antioxidant activity by phosphomolybdenum assay, ferric-reducing antioxidant power (FRAP), ferric ion-chelating and DMPD (*N,N*-dimethyl-*p*-phenylendiamine) radical scavenging activities. All enzyme inhibitory activities and antioxidant assays were tested at initial concentrations of 2000 µg/ml. The activities of the extracts on the α-glucosidase enzyme were also studied at lower concentrations and IC_{50} values were determined, because most of extracts was showed the α-glucosidase inhibitory activity higher than 50% at 2000 µg/ml. Detailed information about the results of activities was given in Tables 2-4.

Total phenol contents of the extracts were measured using Folin Ciocalteu method and expressed as Gallic acid equivalents (GAE). According to our results, total phenol contents of the extracts varied from 90.05 ± 3.73 to 193.67 ± 7.89 mg GAE/g extract (Table 1). Consequently, there seems to be no correlation between the total phenol content of extracts and enzyme inhibitory and antioxidant activity except DMPD radical scavenging activity.

Table 1. Yield percentages (w/w), total phenolic contents (mg GAE/g extract) of the extracts and parts of the plants.

Plant	Part used	Yield (w/w)	Total Phenolic Content (mg/g) GAE ± SEM[a]
Lamium purpureum var. *purpureum*	Aerial part	20.27	93.50 ± 1.44
Origanum onites	Aerial part	22.96	112.37 ± 8.02
Salvia sclarea	Leaf	19.15	123.06 ± 21.15
Salvia sclarea	Flower	28.51	90.05 ± 3.73
Salvia virgate	Aerial part	15.04	193.67 ± 7.89
Thymus zygioides var. *lycaonicus*	Aerial part	10.53	193.47 ± 4.45

GAE: Gallic acid equivalent [a] Standard error of the mean (n=3)

α-Glucosidase inhibitory activity results showed that the extract of *T. zygioides* var. *lycaonicus* was found to have the highest inhibitory activity with 546.7 µg/ml IC_{50} value among all tested plant extracts (Table 3). α-Glucosidase inhibitory activities of the other extracts followed the order: *O. onites* > *S. sclarea* (leaf) > *S. sclarea* (flower) > *S. virgata* > *L. purpureum* var. *purpureum*. Acarbose used as reference exerted strong α-glucosidase inhibitory activity with IC_{50} value of 181.4 µg/ml (Table 3). On the other hand, α-amylase inhibitory activity

of all tested extracts was found to be extremely weak (2.30 ± 0.21-8.93 ± 1.73 %). *L. purpureum* var. *purpureum* extract was found to be inactive at tested concentration.

Except for *L. purpureum* var. *purpureum, S. virgata* and *T. zygioides* var. *lycaonicus* extracts, the rest of the extracts showed moderate activities (20.72 ± 2.08-24.34 ± 1.90 %) on pancreatic lipase. In this study, orlistat that is used for the management of obesity inhibited pancreatic lipase enzyme with 64.53 ± 0.32 % at 1 µg/ml (Table 2).

Table 2. Inhibitory activity of the extracts against α-glucosidase, α-amylase, pancreatic lipase, AChE and BChE enzymes at 2000 µg/ml concentration

Plant	Inhibitory activity (% ± SEM[a])				
	α-Glucosidase	α-Amylase	Pancreatic lipase	AChE	BChE
L. purpureum var. *purpureum*	46.75 ± 1.54***	-	-	-	8.52 ± 0.61***
O. onites	77.39 ± 0.76***	3.16 ± 0.99 ns	20.72 ± 2.08*	-	15.56 ± 1.69***
S. sclarea (Leaves)	72.95 ± 1.04***	2.30 ± 0.21 ns	24.23 ± 0.18**	-	51.76 ± 1.04***
S. sclarea (Flowers)	64.72 ± 1.06***	8.07 ± 0.15*	24.34 ± 1.90**	-	31.91 ± 1.01***
S. virgata	61.15 ± 2.03***	8.93 ± 0.10**	-	-	-
T. zygioides var. *lycaonicus*	85.28 ± 0.89***	8.93 ± 1.73 ns	-	-	30.92 ± 1.44***
Reference	85.30 ± 0.46[b]***	91.00 ± 0.43[b]***	64.53 ± 0.32[c]***	95.50±1.31[d]***	87.51 ± 0.24[d]***

[a] Standard error of the mean (n=3), [b]Acarbose 2000 µg/ml, [c]Orlistat 1 µg/ml, [d]Galanthamine 1000 µg/ml

The inhibitory activity results of the extracts on cholinesterase enzymes displayed that the extracts had no AChE inhibitor activity. Conversely, the highest BChE inhibitor activity was seen in *S. sclarea* leaf extract with 51.76 % value. *S. sclarea* flower and *T. zygioides* var. *lycaonicus* extracts also showed moderate BChE inhibitor activities (31.91 %). Only *S. virgata* extract was found to be inactive against BChE enzyme. In this assay, galantamine used as reference inhibited BChE with 87.51 ± 0.24 % at 1 mg/ml (Table 2).

Table 3. The IC_{50} value of α-glucosidase inhibitory activity of the extracts.

Plant	IC_{50} (µg/mL ± SEM[a])
L. purpureum var. *purpureum*	>2000
O. onites	814.00 ± 5.02
S. sclarea (Leaves)	877.87 ± 15.34
S. sclarea (Flowers)	1075.00 ± 44.51
S. virgata	1113.67 ± 36.91
T. zygioides var. *lycaonicus*	565.20 ± 7.92
Acarbose	0.33 ± 0.03

[a] Standard error of the mean (n=3)

Total antioxidant activity determined by phosphomolybdenum assay and expressed as ascorbic acid equivalents (AAE). The *Salvia* species showed the highest total antioxidant activity (209.72 ± 2.35 – 490.20 ± 1.16 AAE). FRAP assay is one of the methods used to evaluate the antioxidant activity. The extracts (1.12 ± 0.08 - 1.17 ± 0.06) were found to have better antioxidant effects compared to reference compound quercetin (0.97 ± 0.10). In the DMPD radical scavenging assay, ascorbic acid (69.46 ± 2.14 %) showed a potent activity while the extracts showed little or no activity. Except for *L. purpureum* var. *purpureum* (18.42 ± 1.17 %), all extracts were inactive on ferric-ion chelating activity assay (Table 4).

Table 4. Ferric reducing antioxidant power (FRAP), ferric ion-chelating and DMPD radical scavenging activity.

Plant	Total antioxidant (AAE ± SEM[b])	FRAP[a] (Abs. at 700 nm ± SEM[b])	Ferric ion-chelating act. (% ± SEM[b])	DMPD scavenging (% ± SEM[b])
L. purpureum var. *purpureum*	58.21 ± 0.85	1.17 ± 0.06***	18.42 ± 1.17 ns	-
O. onites	44.82 ± 1.10	1.12 ± 0.08***	-	20.53 ± 0.04**
S. sclarea (Leaves)	490.20 ± 1.16	1.34 ± 0.08***	-	20.60 ± 8.69**
S. sclarea (Flowers)	209.72 ± 2.35	1.34 ± 0.08***	-	7.87 ± 1.76 ns
S. virgata	225.23 ± 0.68	1.27 ± 0.01***	-	30.12 ± 1.29**
T. zygioides var. *lycaonicus*	17.34 ± 1.06	1.29 ± 0.08***	-	22.57 ± 0.73**
Reference		0.97 ± 0.10[c]***	94.98 ± 0.06[d]	69.46 ± 2.14[e]***

[a] Higher absorbance indicates greater antioxidant activity, [b] Standard error of the mean (n=3), [c] Quercetin 1000 µg/ml, [d] EDTA 2000 µg/ml, [e] Ascorbic acid 1000 µg/ml, AAE: Ascorbic acid equivalent.

3. DISCUSSION

Medicinal plants are extremely good resources for finding new drug molecules for the treatment of DM and Alzheimer's disease. In this report, we tried to evaluate antioxidant, antidiabetic, anticholinesterase, and antiobesity activities of five Lamiaceae plants. The results of the *in vitro* activity tests have shown that detailed studies are needed on some of the tested species.

The α-glucosidase inhibitory activity results have been supported the traditional use of *O. onites* and *T. zygioides* var. *lycaonicus* in DM treatment. In previous studies, it is demonstrated that *O. majorana*, *O. vulgare* ssp. *hirtum, T. satureioides*, *T. praecox* subsp. *skorpilii* var. *skorpilii* and *T. vulgaris* also exhibited antidiabetic activity in experimental diabetic rats and *T. capitatus* showed α-amylase inhibitory activity [16, 20-24].

According to the results of an *in vivo* antidiabetic study, it was thought that Salvianolic acid B is the major bioactive compound in *S. miltiorrhiza* [25]. The antidiabetic activities of *S. officinalis*, *S. fruticosa*, *S. lavandulifolia, S. triloba* and *S. hispanica* were also exhibited [16, 26-30]. With bioactivity guided isolation technique, the α-amylase inhibitory activity of *S. virgata* was attributed to chrysoeriol by Nickavar and Abolhasani [31]. As a result of bioassay guided isolation studies to date, different structured compounds (5-hydroxy-7,4'-dimethoxyflavone, amarisolide, pedalitin, apigenin-7-*O*-β-D-glucoside, the flavone 2-(3,4-dimethoxyphenyl)-5,6-dihydroxy-7-methoxy-4*H*-chromen-4-one, luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide, diosmetin-7-*O*-glucuronide, (1*R*, 5*S*, 7*S*, 8*S*, 9*R*, 10*R*, 12*R*)-1,7,8-trihydroxycleroda-3,13(16),14-triene-17,12;18,19-diolide, and oleanolic acid) exhibiting α-glucosidase inhibitory activity were isolated from *Salvia* species [32-35]. Since many *Salvia* species (*S. triloba, miltiorrhiza, hydrangea, officinalis* etc.) have been found to have *in vitro* and *in vivo* antidiabetic activity, *S. virgata* and *S. sclarea* extracts taken into this study and have been consistently found to have strong α-glucosidase enzyme inhibitory activity.

On the other hand, *S. miltiorrhiza* also exerted protective activity against memory and learning impairments in streptozotocin-induced diabetic rats [36-38]. Likewise, the protective effects of *S. officinalis* against memory and learning deficiency induced by diabetes was shown and this activity was attributed to rosmarinic acid [39].

On the other hand Şenol *et al*. [17] studied AChE inhibitory activity of 55 different taxa of *Salvia.* Among the tested extracts, only *S. fruticosa* showed relatively significant AChE inhibitory activity. In these study at 25, 50 and 100 µg/ml of the ethyl acetate and dichloromethane extracts of *S. virgata*, which was collected from Ankara, Turkey and it has been observed that the extract does not have any enzyme inhibitory effect compared with galanthamine (98.97 ± 0.24 % at 100 µg/ml). In our study, among the *Salvia* species tested, it was also found that *S. virgata* ethanolic extract, which was collected from Isparta, Turkey did not show any inhibitory activity on both AChE and BChE enzymes, whereas the leaf ethanol extract of *S. sclarea* had strong BChE inhibitory activity. In another study, which was conducted by Kindl *et al*., *T. longicaulis*, *T.*

praecox subsp. *polytrichus*, *T. pulegioides*, *T. serpyllum* subsp. *serpyllum*, *T. striatus*, and *T. vulgaris* were demonstrated inhibitory activity against AChE [18]. This activity may be caused from carvacrol, which is one of most important compounds of *Thymus* species [40]. On the other hand, it was displayed that *Origanum majorana* has strong AChE activity and the active compound of *O. majorana* was found as ursolic acid [19]. As Topçu *et al*. [41] emphasized, the Lamiaceae is family rich in species with potent anticholinesterase activity.

This literature data support that there is no relationship between the total phenol content of our tested extracts and their anticholinesterase inhibitory activity.

Oxidative stress plays an important role in the pathogenesis of many neurodegenerative diseases and DM. Therefore, the plant extracts having antidiabetic or anticholinesterase activity are also expected to show antioxidant activity. The *Salvia* species (*S. sclarea*, and *S. virgata*) exhibited more total antioxidant activity when compared to the other extracts. In this investigation, among the tested antioxidant activities, we have noticed that all extracts have strong FRAP activities. The reducing properties of the test samples were measured by this method. These samples having reduction potential donate an electron to free radicals and convert them into more stable forms. And thus, free radicals that convert to this stable form can be prevented from causing membrane lipid peroxidation. This mechanism prevents the deterioration of cell membrane stability caused by excess free radicals occurring in Alzheimer and DM.

Although *T. zygioides* var. *lycaonicus* was used traditionally against diabetes, no previous studies of antidiabetic and antialzheimer activities of *T. zygioides* var. *lycaonicus* have been found. This is the first study on enzyme inhibitory effects of *T. zygioides* var. *lycaonicus*. The activity of *O. onites* essential oils was indicated against cholinesterase enzymes by Orhan *et al*. [42], but the inhibitory activity of aerial part of *O. onites* and *L. purpureum* var. *purpureum* against α-amylase, α-glucosidase, pancreatic lipase, and cholinesterase enzymes was demonstrated in this study for the first time.

4. CONCLUSION

The results of the study indicated that except for *L. purpureum* var. *purpureum*, all tested plants herein belonging to Lamiaceae family have shown inhibitory effects against the α-glucosidase. Significant activity against pancreatic lipase was observed only with *S. sclarea* and *O. onites* extracts. In addition, the fact that all the extracts tested had strong FRAP activities suggest that they would provide significant protection against oxidative stress in DM and AD. Further studies should be focused on the BChE inhibitory activity of *S. sclarea* flower and *T. zygioides* var. *lycaonicus* aerial part extract and α-glucosidase inhibitory activity of *T. zygioides* var. *lycaonicus*.

5. MATERIALS and METHODS

5.1. Plant materials

The aerial parts of *Lamium purpureum* var. *purpureum*, *Origanum onites* were collected in May 2015, the herbs of *Salvia virgata*, *Thymus zygioides* var. *lycaonicus*, leaves and flowers of *S. sclarea* were collected in June 2014 from Isparta Turkey. The plants were identified by Dr. Ufuk ÖZBEK (Gazi University, Faculty of Science and Arts, Department of Biology, Ankara) and Dr. Galip AKAYDIN (Hacettepe University, Faculty of Science and Arts, Department of Biology, Ankara). Herbarium specimens are stored in the Herbarium of Faculty of Pharmacy Gazi University, Ankara, Turkey (GUEF 3439, GUEF 3445, GUEF 3431, GUEF 3447, GUEF 3429, respectively).

5.2. Extraction

The dried and powdered plant materials (10 g) were extracted with ethanol (80%, 200 ml) on a shaker. After filtration, the extracts were concentrated under vacuum (at 40 °C) by evaporation to dryness. The extraction was repeated for three times and the dried extracts were pooled. The yields of the extracts are given in Table 1. For the determination of enzyme inhibitory activities, in order not to affect enzyme activities, the extracts were dissolved in DMSO (3%), and for antioxidant activities was dissolved in ethanol (80%).

5.3. Enzyme inhibitory activities

5.3.1. Cholinesterase inhibitory activity

AChE and BChE inhibitory activities of the extracts were measured by slightly modifying the spectrophotometric method developed by Ellman et al. [43]. Horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) and electric eel AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) enzymes were used as

enzyme sources. Butyrylthiocholine chloride (BChC) and acetylthiocholine iodide (AChI) (Sigma, St. Louis, MO, USA) were used as substrates. First of all, 20 µl of test solution, Ellman's reagent, and enzyme solution were added in sodium phosphate buffer (pH 8.0) and incubated for 15 min at 25ºC. The reaction was started with the addition of substrate and the substrate was hydrolyzed to thiocholines. As a result of the reaction of thiocholines with Ellman's reagent yellow colored 5-thio 2-nitrobenzoate anion occurs. Change in absorbance (at 412 nm), utilizing a 96-well microplate reader (VersaMax Molecular Devices, Sunnyvale, CA, USA). Percentage inhibition of the enzymes was determined by using the formula: Inhibition (%)= (B-S)/Bx100 where B is the activity of the enzyme without test sample and S is the activity of the enzyme with a test sample. The experiments were done in triplicate. Galanthamine (Sigma, St. Louis, MO, USA) was used as the reference.

5.3.2. α-Amylase inhibitory activity

The α-amylase inhibition method was performed according to the method by Ali et al. [44] with slight modification. For the preparation of enzyme solution, porcine pancreatic α-amylase (EC 3.2.1.1, type VI, Sigma) was dissolved in ice-cold distilled water (4 U/ml). Potato starch (0.5 %, w/v) in 20 mM phosphate buffer (pH 6.9) was used as substrate solution. Plant extract and enzyme solution were mixed in an Eppendorf tube. The tubes were incubated at 37°C for 5 min. Then the starch solution was added and the reaction was started. Then DNS color reagent solution (5.31 M sodium potassium tartrate in 2 M NaOH and 96 mM 3,5-dinitrosalicylic acid) was added and heated at 85°C. Tubes were cooled immediately and the absorbance of the mixture was read at 540 nm. Acarbose was used as a reference. Experiments were performed with three replicates. The absorbance (A) according to the produced maltose is calculated with the following equation (Eq. 1):

$$A_{\text{control or plant extract}} = A_{\text{Sample}} - A_{\text{Blank}} \quad \text{(Eq. 1)}$$

The amount of produced maltose was determined by the calibration curve of maltose standard. Inhibition percentage was determined as: Inhibition % = (mean maltose in control-mean maltose in sample)/mean maltose in control × 100.

5.3.3. α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity was carried out in accordance with the method of Lam et al. [45]. Enzyme solution of α-glucosidase type IV (Sigma Co., St. Louis, USA) from *B. stearothermophilus* was prepared with dissolving the enzyme in phosphate buffer (pH 6.5). Extracts were preincubated with enzyme solution for 15 min at 37 °C. 4-Nitrophenyl β-D-glucopyranoside (Sigma) as substrate (20 mM) was added and the incubation proceeded for 35 min at 37 °C. The substrate hydrolyzed with the enzyme to *p*-nitrophenol (yellow) and the resulting absorbance was measured with an Elisa plate reader (Versamax Tunable Microplate Reader) at a wavelength of 405 nm. Acarbose (Bayer) was used as reference. The inhibition percentage (%) was calculated according to this formula: Inhibition (%)= (B-S)/Bx100 where B is the activity of the enzyme without test sample and S is the activity of the enzyme with a test sample.

5.3.4. Pancreatic lipase inhibitory activity

The inhibitory activities of the plant extracts on pancreatic lipase were determined by the method used by Lee et al. [46]. For the preparation of enzyme solution, porcine pancreatic lipase enzyme type II (Sigma) dissolved in a buffer (pH 6.8) contains 10 mM MOPS (4-Morpholinepropanesulfonic acid, Sigma) and 1 mM EDTA. Enzyme solution and extracts were mixed in Tris buffer (100 mM Tris–HCl and 5 mM $CaCl_2$, pH 7.0) and preincubated for 15 min at 37°C. Then substrate solution (5 mM *p*-nitrophenyl butyrate in dimethyl formamide) was added and proceed for 30 min at 37°C. Lipase activity was determined by reading the absorbance generated by *p*-nitrophenol hydrolysis to *p*-nitrophenyl butyrate with an ELISA reader (Versamax Tunable Microplate Reader) at 405 nm. Orlistat (Roche) was used as reference. Lipase inhibition (%) was determined according to this formula: Inhibition (%)= (B-S)/Bx100 where B is the activity of the enzyme without test sample and S is the activity of the enzyme with a test sample.

5.4. Antioxidant activity assays

5.4.1. Total antioxidant activity by phosphomolybdenum assay

Total antioxidant activity was conducted by Prieto et al. [47]. In the assay, Mo (VI) reduced to Mo (V) by the extracts and then the green phosphate/Mo (V) complex occurs at acidic pH. The extracts were added

to tubes containing distilled water and molybdate reagent solution (Molybdate reagent: sodium phosphate (28 mM), sulphuric acid (0.6 M), and ammonium molybdate 4 mM). Tubes were vortexed, followed by were incubated for 90 min at 90°C. Tubes were cooled to room temperature and the absorbance of the mixture was determined at 695 nm. The results were expressed as mg AAE/g extract.

5.4.2. DMPD radical scavenging activity

DMPD (N,N-dimethyl-*p*-phenylendiamine) radical scavenging activity was determined by the method of Schlesier et al. [48] 100 mM DMPD and 0.05 M ferric chloride solutions prepared freshly and were involved with 0.1 M acetate buffer (pH=5.25). The absorbance of reagent at 505 nm was equilibrated to 0.900±0.100. After that, 50 µl from each extract added to the reagent and absorbance of the reaction mixture was determined at 505 nm. Ascorbic acid was used as reference.

5.4.3. Ferric ion-chelating activity

The metal chelating activity of all extracts was determined by the method of Chua et al. [49]. 20 µl extract mixed with 2 mM $FeCl_2$. Then 5 mM ferrozine solution added to the mixture and the reaction was started. After 10 minutes passed, the absorbance of the reaction mixture was determined at 562 nm. EDTA was used as reference. The inhibition of Fe^{+2}-ferrozine complex establishment (%) was determined according to this equation (Eq. 2):

$$\text{Inhibition (\%)} = (A_{control}-A_{sample})/A_{control}x100 \quad \text{(Eq. 2)}$$

5.4.4. Ferric-reducing antioxidant power (FRAP)

2.5 ml of potassium ferricyanide and plant extracts were added into 2.5 ml of phosphate buffer (pH 6.6). The reaction proceeded for 20 min at 50 °C. Then trichloroacetic acid (10 %), $FeCl_3$ (0.1 %, w/v) were added. After the 30 min of incubation, the absorbance of the mixture was determined at 700 nm. Increase in absorbance of the reaction indicated ferric-reducing antioxidant power of the extracts [50].

5.5. Total phenolic content

Folin-Ciocalteu method [51] was used to determine the total phenolic content. The plant extract is dissolved in ethanol (75 %) as the concentration is 2 mg/ml. Folin-Ciocalteau and 20 % sodium carbonate solution are added to the extracts. Dilutions of gallic acid are prepared at concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml, 250 mg/ml and 500 mg/ml in order to form the calibration curve. All tubes are allowed to incubate at 40 ° C for 30 min. At the end of the time, the absorbance is read on the spectrophotometer at 765 nm wavelength. According to the calibration curve prepared with the gallic acid solutions, the total phenolic concentration is calculated as the GAE using the absorbance of the sample solution Equation (Eq. 3) of the calibration graph was found as presented below:

$$y = 2.4632x - 0.0419 \text{ and } r^2: 0.9935 \quad \text{(Eq. 3)}$$

5.6. Statistical analysis

Statistical analysis of enzyme inhibition was performed by applying oneway ANOVA, Dunnett multiple comparison test with GraphPad Prism 6.0 program. In the mean p values of $p<0.05$ was considered as significant ($^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, $^{ns}p>0.05$). All experiments were studied in triplicate. The mean and the standard error of the mean values (n=3) were determined with the Microsoft Excel program.

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