Journal of Applied Pharmaceutical Science Vol. 12(08), pp 137-147, August, 2022
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2022.120814
ISSN 2231-3354

UHPLC-ESI-MS analysis of Javanese *Tamarindus indica* leaves from various tropical zones and their beneficial properties in relation to antiobesity

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ARTICLE INFO

Received on: 21/12/2021
Accepted on: 11/05/2022
Available Online: 04/08/2022

Key words:
Köppen–Geiger climate zone, *Tamarindus indica*, UHPLC-ESI-MS, antiobesity, lipase inhibitory.

ABSTRACT

Numerous therapeutic strategies for treating and preventing obesity have been proposed. Pharmacotherapy with orlistat, sibutramine, or antagonists of cannabinoid receptors still has some side effects. *Tamarindus indica* leaves are said to have lipid-lowering properties, suggesting that they could be used as antiobesity agents. The purpose of this study is to analyze the phytochemicals of the tamarind leaf extract grown in a variety of climates and to determine its antilipase and antiamylase activity. The phytochemical profile of T. indica leaf extracts from various tropical zones was investigated using ultrahigh-performance liquid chromatography in conjunction with electrospray ionization high-resolution mass spectrometric analysis (UHPLC-ESI-MS). Followed by metabolite annotation using Compound Discoverer 3.2 software. Antiobesity-related properties were measured including antioxidant capacity, antilipase and antiamylase activity, total phenolic and flavonoid content, and vitexin level of the extract. The enzyme pancreatin and p-nitrophenylbutyrate were used to assess the antilipase activity. Meanwhile, an antiamylase assay was carried out by measuring starch hydrolysis by pancreatin in the presence of extract. sThe results indicated that the antioxidant activity of the tamarind leaf extract was more proportional to the total phenol content ($r = 0.998$) than to the total flavonoids ($r = -0.379$) as determined by the 2,2-diphenyl-1-picrylhydrazyl assay. Tamarind leaves grown in tropical monsoon climates were found to be significantly capable of inhibiting lipase enzymes at 3.8 µg/ml ($p = 0.0026$). Along with flavonoids, it is believed that sesquiterpenes, alkaloids, and steroids contribute to the high synergistic lipase inhibition activity. According to enzyme kinetics analysis, the pattern of inhibition resembled that of mix-mode inhibition.
This information is beneficial for standardizing the tamarind leaf extract for use in the development of herbal medicines. Further research is recommended, particularly to determine the extract's activity against other enzymes or proteins implicated in obesity, such as lipoprotein lipase, leptins, and phosphatidic acid phosphatase.

INTRODUCTION

Obesity is a global problem that is largely regarded as one of the greatest hazards to global health in the modern period (NHS, 2019). Worldwide, around 2 billion individuals are overweight, with 650 million of them clinically obese (WHO, 2021). There are at least two types of drugs available to treat obesity at the moment. The first class of drugs (orlistat) suppresses pancreatic lipase, resulting in decreased intestinal fat absorption. The second is based on appetite suppressants such as sibutramine. Both medications, however, have a number of adverse effects, including constipation, hypertension, xerostomia, headaches, and insomnia (Scheen, 2010; Westerink and Visseren, 2011). As a result, the hunt for a more effective and safe approach to reducing obesity continues. While it is common to use numerous traditional herbal medications on a daily basis for obesity surveillance, their effectiveness and safety have been little explored and have not been convincingly demonstrated in this context.

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It is well established that digestive enzymes such as pancreatic lipase and amylase are responsible for the hydrolysis of triacylglycerols and carbohydrates into simple molecules (Tucci, 2010). Additionally, naturally occurring polyphenols inhibit certain digestive enzymes, which can regulate the caloric content of food by reducing its absorption and lengthening the digestive process (Wang *et al*., 2020). This way, weight loss may become a feasible goal, resulting in significant health benefits.

Numerous studies have emphasized the health benefits of traditional herbal medicine. *Tamarindus indica* was singled out for its overall wellbeing effects. It is an evergreen tropical shrub that grows plentifully in Indonesia. According to an ethnomedicine study, this leaf was used by Indonesians as a component of a traditional herbal medicine known as *jamu sinom*, which translates as "herbal drink for body refreshment" (Handayani *et al*., 2001). Tamarind is a member of the Fabaceae family, more precisely the subfamily Caesalpiniaceae. Its leaves contain limonene, caryophyllene, beta-sitosterol, linalool, malic acid, palmitic acid, tartaric acid, and flavonoids (luteolin and apigenin) (Escalona-Arranz *et al*., 2010). Although the tamarind fruit extract was claimed to lower blood LDL (low density lipoprotein) cholesterol levels when given as tamarind fruit pulp, a clinical trial found that triglyceride levels increased unexpectedly (Iftekhar *et al*., 2006). The polysaccharide content of the fruit could have contributed to the increase in TG. The tamarind leaf extract is expected to have a greater antihyperlipidemic effect than the tamarind fruit extract due to its lower polysaccharide content. According to Nofianti *et al*. (2019), the tamarind leaf extract was effective in reducing hyperlipidemia in rats *in vivo* and also exerted glucose-lowering activity (Yerima *et al*., 2014).

Tamarindus indica was previously investigated in terms of metabolites extracted using a variety of solvents (Escalona-Arranz *et al*., 2011). In this context, and motivated by widespread public use of traditional herbal medicine derived from *T. indica* and its well-documented biological properties, this study focused on the phytochemical constituents of three distinct tropical zones. To aid in the dereplication of metabolites, a mass spectral analysis from ultrahigh-performance liquid chromatography-Electrospray ionization-mass spectrometry (UHPLC-ESI-MS) was performed. Simultaneously, the inhibiting effects of three *T. indica* leaf extracts from various zones on pancreatic lipase and amylase enzymes were examined for their potential use in the treatment of obesity.

MATERIALS AND METHODS

Plant material

As it is known that the water content in the soil affects the production of secondary metabolites, several *T. indica*-producing areas were selected on the Island of Java, which has different rainfall and evaporation rates. *Tamarindus indica* leaves were collected from farmers in three distinct tropical zones on Java Island based on the Köppen–Geiger climate classification map for Indonesia (1980–2016) (Fig. 1), namely rainforest denoted by R [Cianjur district; minimum temperature (T_{min}) ≥ +18°C, precipitation on driest season (P_{driest}) ≥ 60 mm); monsoon denoted by M (Sleman district; T_{min} ≥ +18°C, P_{annual} ≥ 25 mm); and savannah denoted by S (Gresik district; T_{min} ≥ +18°C, P_{driest} < 60 mm). The plants obtained were identified at the Department of Pharmaceutical Biology, Gadjah Mada University.

Chemicals

Pancreatin (P3292-25G), vitexin standard, gallic acid, quercetin, phosphate buffer saline (PBS) tablet, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, nitrophenol glucose, dinitrosalicylic acid, and *p*-nitrophenylbutyrate (N9867-1G) were obtained from Sigma-Aldrich (St. Louis, MO). Orlistat (Xenical) was obtained from a local distributor. The solvent for LC-HRMS (liquid chromatography - high resolution mass spectroscopy) was MS grade purchased from Thermo Scientific™.

Preparation of the extracts

Fresh leaves were dried for 24 hours at 50°C in a tray oven before being powdered with a grinder. Maceration of the leaf powder in 70% ethanol at room temperature (25 ± 2°C) was performed for 24 hours. After filtration, a rotary evaporator was used to evaporate the filtrate into dryness.

Phytochemical contents: UHPLC-ESI-MS

The phytochemical content of the tamarind leaf extract was analyzed using the untargeted mode of the Q Exactive Orbitrap UHPLC-ESI-MS System (Thermo Scientific™). The extract was dissolved in 100 µg/ml MS grade methanol and filtered through a 0.22 µm filter. The device was set up as follows: the mobile phases were (A) aqueous formic acid 0.1% and (B) acetonitrile-formic acid (0.1%); injection volume was 5 µl and flow rate was 0.3 ml/minute. The gradient system was 0–16 minutes (B 5%–90%); 16–20 minutes (B 90%); 20.1–25 minutes (B 5%). Ion source ESI 3.62 kV-positive mode was used. Furthermore, the raw chromatograms were analyzed using the Compound Discoverer 3.2 (Thermo Scientific™) software with reference to local and online databases, including mzCloud (www.mzcloud.org) and ChemSpider (www.chemspider.com).

Determination of total phenolic content

Measurement of the total phenolic content was carried out by mixing 14 µl of extract (500 µg/ml in methanol), 208 µl of distilled water, and 14 µl of the *Folin*–Ciocalteu *reagent*. The mixture was incubated for 8 minutes. After incubation, the mixture was added with 14 µl of Na_2CO_3 (20%) and then incubated for 1.5 hours, followed by absorbance measurement at 765 nm. Gallic acid was used as a standard curve (series of 20–150 µg/ml). Total phenolic content was expressed as milligrams gallic acid equivalent per gram of extract (Wiyono *et al*., 2020).

Determination of total flavonoid content

The total flavonoid content was measured using a spectrophotometric method with an aluminum chloride reagent (Chandra *et al*., 2014). A total of 0.8 ml of extract (500 µg/ml in methanol) was mixed with 0.8 ml of 2% $AlCl_3$. After incubation for 60 minutes, the absorbance of the solution was measured at 420 nm. Quercetin (20–150 µg/ml) was used as the standard. Flavonoid content was expressed as milligrams of quercetin equivalent per gram of extract.

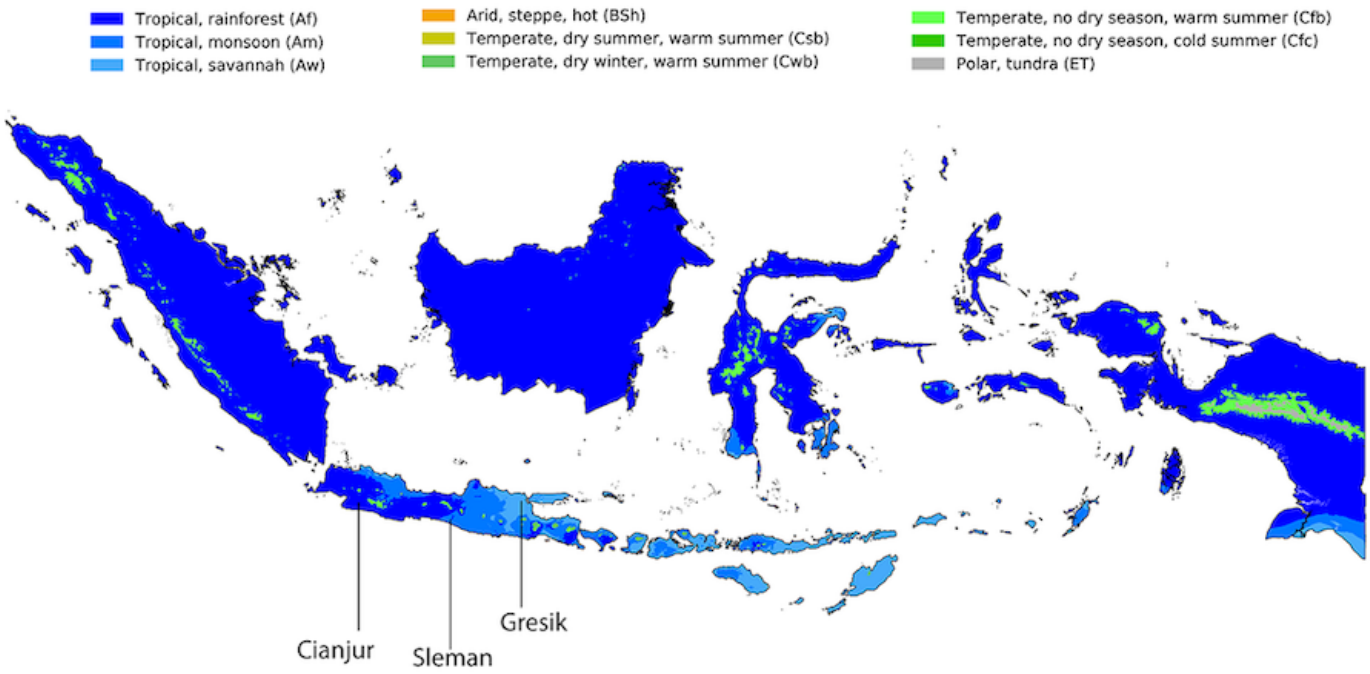

Figure 1. Koppen–Geiger climate classification map for Indonesia (1980–2016) and sources of experimental plant. Cianjur represented Rainforest-zone, Sleman as Monsoon-zone, and Gresik as Savanah-zone. (Beck *et al*., 2018).

Determination of vitexin content

Vitexin levels in the tamarind leaf extract were determined using the Q Exactive Orbitrap UHPLC-ESI-MS System in the targeted mode (Thermo Scientific™). The extract was dissolved in 100 µg/ml MS grade methanol and filtered through a 0.22 µm filter. The device was initially configured as follows: the mobile phase was (A) aqueous formic acid 0.1% and (B) acetonitrile-formic acid (0.1%); injection volume was 8 µl; flow rate was 0.3 ml/minute. Gradient system was 0–8 minutes (B 5%–90%); 8–13 minutes (B 90%); 13.01–18 minutes (B 5%). The ion source used was electrospray ionization in positive mode (3.32 kV). The vitexin standard was prepared by dissolving vitexin in methanol of MS grade in a series of concentrations of 0.5–20 µg/ml.

DPPH radical scavenging assay

Because the measurement of IC_{50} DPPH scavenging is often biased, depending on the concentration of DPPH used as mentioned in a previous critical review (Menezes *et al*., 2021), the measurement of antioxidant capacity was used in this study instead of IC_{50}. A volume of 0.5 ml of the tamarind leaf extract at 500 µg/ml (in methanol) or ascorbic acid (in methanol) at concentrations ranging from 20 to 100 µg/ml was mixed with 0.5 ml of DPPH (1,1-diphenyl-2-picrylhydrazyl) at 0.04 mg/ml (in absolute ethanol) and stored at room temperature in a dark place. After 30 minutes of incubation, the absorbance at 520 nm was measured. The extract's antioxidant capacity was expressed as gram ascorbic acid equivalent per 100 g of extract.

Pancreatic lipase inhibition

The lipase inhibitory activity was determined spectrophotometrically by monitoring the rate of nitrophenol formation as a product of pancreatin's hydrolysis of *p*-nitrophenylbutyrate (Terra *et al*., 2016). The extract was prepared by dissolving the extract in DMSO (dimethyl sulfoxide) at concentrations of 10 and 100 µg/ml. About 10 µl of each extract solution (for final mixture concentrations of 0.38 and 3.8 µg/ml, respectively) were mixed with 10 µl of pancreatin (1 mg/ml in phosphate buffer saline, pH 6.8). After 5 minutes of incubation at 37°C, the substrate 240 ml (*p*-nitrophenylbutyrate 0.165 mM in PBS) was added. The absorbance was measured at 415 nm immediately after substrate addition and 35 minutes later. The lipase activity was expressed as µM nitrophenol released per minute. Orlistat 120 µg/ml (4.6 µg/ml final concentration) was used as a positive control. Nitrophenol standard curves were created by diluting nitrophenol to concentrations of 0, 1, 2, 5, 10, and 20 µg/ml. About 260 µl of each dilution was measured in a 96-well plate at 415 nm.

To ascertain the mode of inhibition, the enzyme kinetics values (K_m and V_{max}) were determined using the extract and orlistat (final concentrations of 3.8 and 4.6 µg/ml, respectively) at a range of substrate concentrations of 0.1, 0.2, 0.3, and 1.0 mM.

Pancreatic amylase inhibition

Tests on the inhibitory activity of the extract against the amylase enzyme were carried out by the sugar reducing method using pancreatin (1 mg/ml) in phosphate buffer saline of pH 6.8 referring to the method of Keharom *et al*. (2016) with modifications. The starch as amylase substrate was dissolved in distilled water by heating until it was clear. In summary, 50 µl of the extract in DMSO was mixed with 100 µl of the enzyme pancreatin, followed by 2,000 µl of the starch substrate (5,000 µg/ml). The mixture was incubated for 10 minutes at 37°C. Then the reaction was stopped by adding 500 µl of 1 M NaOH, followed by the addition of 200 µl dinitrosalicylic acid 2.75 mg/ml. The mixture was then heated in

boiling water for 5 minutes. Absorbance reading was carried out by taking 100 µl of the solution that was diluted with 1,000 µl of distilled water in a cuvette and then measured at 410 nm. As the standard curve, standard glucose (serial concentration 2.5–500 µg/ml) was used.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 7.0 with the one-way analysis of variance followed by Dunnett's multiple comparison test. Pearson's correlation test was performed to determine the correlation between antioxidant activity and phytochemical level. Differences among comparisons were considered statistically significant for p values less than 0.05 (95% confidence level). Nonlinear regression analysis was also carried out to calculate the enzyme kinetics value (K_m and V_{max}). The data are presented as mean±standard deviation.

RESULTS

Effect of various climate zones on total phenolic, flavonoid, and vitexin content

The gallic acid standard curve $y = 0.0014x + 0.0501$ ($r^2 = 0.9912$) was obtained to determine total phenolic content. Meanwhile, the quercetin standard curve was $y = 0.0192x + 0.0786$ ($r^2 = 0.9992$). The results indicated that tamarind leaves from the monsoon zone (M) had the highest total phenolic content (55.9 ± 1.9% GAE, gallic acid equivalent), which was proportional to their antioxidant activity (r = 0.998). Meanwhile, the highest total flavonoid content (2.1 ± 0.0% QE, quercetin equivalent) was owned by tamarind leaves grown in the savannah climate zone (S). The flavonoid content was poorly correlated ($r = -0.379$) to antioxidant activity based on Pearson's correlation test. The total phenolic, flavonoid, and vitexin content are presented in Table 1.

Effect of various climates on vitexin content

The content of vitexin was quantified using targeted UHPLC-ESI-MS. Standard vitexin was injected into HRMS in series, and the peak area was determined, yielding the standard curve $y = 11774802.32x + 1980946.77$ ($r^2 = 0.9985$). As shown in Supplementary Figure 1, both vitexin standard and vitexin in the extract had a retention time of 6.1–6.2 minutes. The results indicated that tamarind leaves from the savannah zone (S) had the highest vitexin content (9.63% of dry extract).

Effect of various climates on antioxidants

The standard curve of ascorbic acid for DPPH scavenging was calculated to be $y = 0.43x - 2.75$ ($r^2 = 0.9543$). The antioxidant capacity of the dry extract was determined by extrapolating the extract's absorbance reading to the standard curve and was expressed as percent ascorbic acid equivalent per dry extract, as shown in Table 1. Antioxidant capacities of the rainforest (R),

Table 1. Total phenolic, flavonoid, vitexin and antioxidant capacity of different source of *Tamarindus indica* leaf extract. Except for vitexin content, values were presented as Mean ± SD, $n = 3$.

Climate zone	TPC gallic acid eq. (%)	TFC quercetin eq. (%)	Vitexin content (‰ dry extract)	Ascorbic acid eq. (%) (DPPH method)
Rainforest (R)	35.9 ± 5[a]	1.9 ± 0.1[a]	8.99	31.3 ± 0.8[a]
Monsoon (M)	55.9 ± 1.6[b]	1.3 ± 0.1[b]	5.41	42.4 ± 0.2[b]
Savanah (S)	52.3 ± 0.4[b]	2.1 ± 0.0[a]	9.63	41.1 ± 1.4[b]
Correlation to antioxidant	$r = 0.998$	$r = -0.379$	-	-

The different superscripted letter at the same column indicates significantly different.

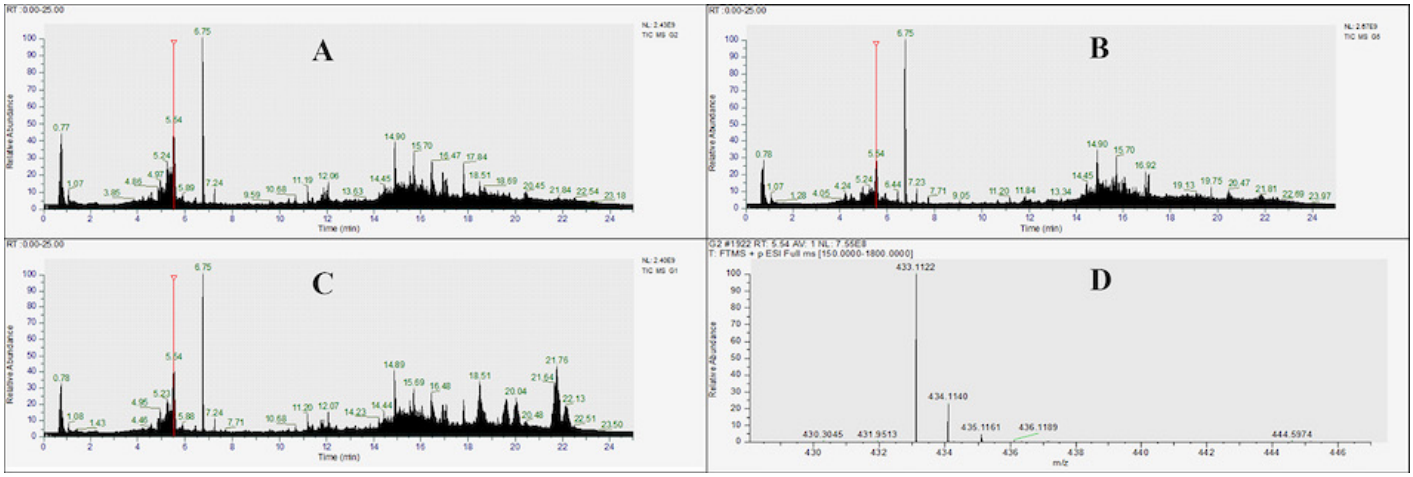

Figure 2. Chromatogram of untargeted LC-HRMS from *T. indica* leaf exract. Label: R (Rainforest), M (Monsoon), S (Savanah), Vtx (MS1 spectra of vitexin).

monsoon (M), and savannah (S) extracts were 31.3%, 42.4%, and 41.1% AAE (ascorbic acid equivalent), respectively.

UHPLC-ESI-MS metabolites annotation

As shown in Figure 3, an analysis of the untargeted LC–MS chromatogram was performed. The analytical parameters for metabolite profile analysis were as follows: candidate molecules were chosen based on their conformation to the fragmentation pattern in mzCloud, and their molecular mass deviation was no greater than 2 ppm. In Table 2, predicted molecules are tabulated and classified according to their compound class.

In general, alkaloids, amides, amino acids, chalcones, coumarins, fatty acids and esters, flavonoids, peptides, phenolic acids and aldehydes, sesquiterpenes, steroids, triterpenoids, and volatile oils were detected in *T. indica* leaf extract. Based on the chromatogram shown in Figure 2, it can be seen that compounds with high abundance appeared at a retention time of 14–22 minutes. The flavonoid content was seen dominantly at the retention time of 4.56–8.45 minutes. The presence of steroid and triterpenoid compounds was more detected in *T. indica* from the savannah (S) zone.

Meanwhile, the distinctive compounds that were only detected in each zone are as follows: the rainforest zone: N-metiltriptamin, 7-methoxycoumarin, 3-(3,4-dihydroxyphenyl)-7-hydroxy-8-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] chromen-4-one, nobiletin, and octadecatrienoic acid methyl ester; the monsoon zone: 3-[(1E,3E)-hepta-1,3-dienyl] pentanedioic acid, phloretin, 5α-androstan-3,6,17-trione, nootkatone, jasmone, (2R,5R,6R)-3-[(1E,3E)-hepta-1,3-dien-1-yl]-5,6-dihydroxy-2-(hydroxymethyl) cyclohexan-1-one, and 1,2-cyclohexane dicarboxylic acid diisononyl ester; the savannah zone: argininosuccinic acid, 2,2,6,6-tetramethyl-1-piperidinol, esculetin, benzidine, quercetin-3β-D-glucoside, 3',4',7-trihydroxy flavanone, ursolic acid, 3-hydroxylupenoic acid, and N,N-diethyldodecanamide.

Pancreatic lipase inhibition

The lipase inhibitory activity measurements revealed that the compound with the highest inhibitory activity was M (monsoon zone) at 3.8 µg/ml giving 58.3% inhibition ($p = 0.0026$), followed by R (rainforest) 40.9% ($p = 0.0454$) and S (savannah) 38.6% ($p = 0.0611$), as compared to the solvent control (Fig. 3A) at the $\alpha = 0.05$ significance level. The inhibitory activity of the *T. indica* leaf extract from the monsoon zone was even stronger than orlistat (4.6 µg/ml).

Additionally, the inhibition mode was determined by varying the substrate concentration so that the Michaelis–Menten curve was formed, as shown in Figure 3B. The mode of inhibition was determined by comparing the maximum reaction rate (V_{max}), Michaelis constant (K_m), and Lineweaver–Burk (LB) slope values in the presence and absence of an inhibitor. According to the data obtained, the results showed that all extracts decreased V_{max} and K_m and altered the value of the LB slope, as shown in Table 3.

Pancreatic amylase inhibition

The results of the amylase inhibitory activity test showed that the *T. indica* leaf extract did not significantly reduce the rate of starch hydrolysis by amylase. At 0.38 and 3.8 µg/ml of extract concentration, there was no decrease in the rate of starch hydrolysis by pancreatic amylase enzymes ($p > 0.05$), as shown in Figure 4.

DISCUSSION

Phytochemicals and antioxidants

Obesity develops as a result of an imbalance in calorie intake and activity. Calories enter the body in the form of carbohydrates (4 calories per gram), fat (9 calories per gram), and protein (4 calories per gram). The number of calories consumed is determined by the rate of absorption in the intestinal lumen and the metabolism of food prior to absorption. Carbohydrates are absorbed in the form of monosaccharide molecules, which were previously hydrolyzed by the amylase and alpha-glucosidase enzyme. Protein is absorbed in the form of amino acids, a process that is initiated by proteases. Meanwhile, fat is absorbed in the form of free fatty acids following the lipase enzyme's hydrolysis of triglycerides (Brodkorb *et al*., 2019). Inhibition of these three enzymes, particularly lipase, has recently become a target for obesity prevention.

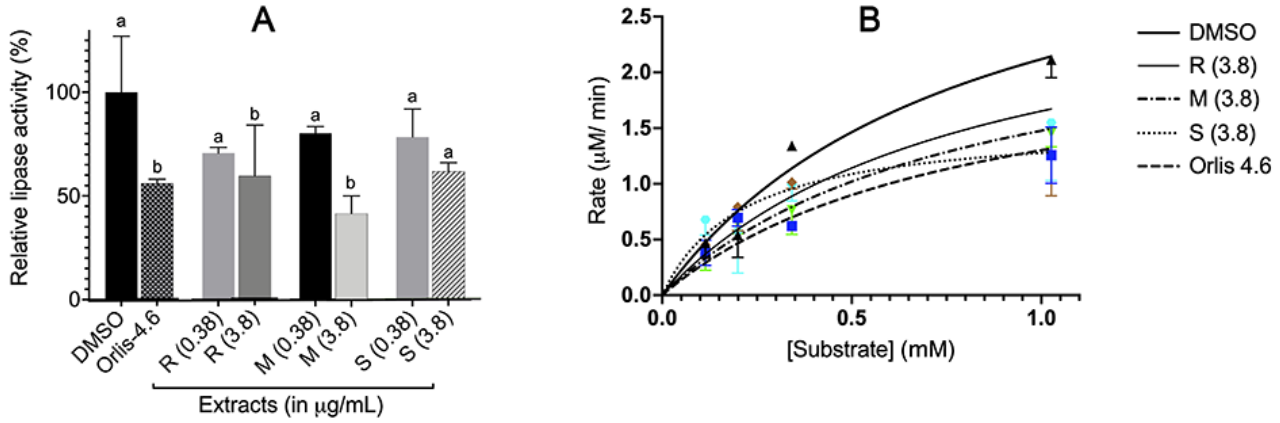

Figure 3. (A) Pancreatic lipase inhibitory activity of *T. indica* leaf extract. The activity was measured using low (0.38 µg/ml) and high (3.8 µg/ml) concentration of extract with 0.165 mM p-nitrophenylbutyrate as substrate. (B) Kinetics of pancreatic lipase in the absent and present of extract.

Table 2. Predicted phytochemical content of *Tamarindus indica* leaf extract using untargeted HRMS method. Sample code: R (rainforest), M (monsoon), S (savanah).

Compound class	Putative name	MW	RT (minute)	R	M	S
Amino acid	L-(+)-Arginine	174.1114	0.713	*		*
Amino acid	Argininosuccinic acid	290.1221	0.752			*
Alkaloid	Adenosine	267.0967	0.776	*	*	*
Amide	Phenacetin	179.0945	0.777	*		*
Alkaloid	Hordenine	165.1152	0.779	*	*	*
Alkaloid	2,2,6,6-Tetramethyl-1-piperidinol	157.1466	0.823			*
Amide	2-(3-methoxyphenyl)acetamide	165.0791	0.837	*		*
Peptide	Prolylleucine	228.1472	1.077	*	*	*
Amino acid	L-Phenylalanine	165.0789	1.404	*	*	*
Alkaloid acid	trans-3-Indoleacrylic acid	187.0631	1.957	*	*	*
Alkaloid	N-Methyltryptamine	174.1156	3.443	*		
Coumarin	7-Hydroxycoumarine	162.0314	3.486	*		
Coumarin	Esculetin	178.0264	3.959			*
Phenolic acid	Ferulic acid	194.0576	4.281	*	*	*
Fatty acid	3-[(1E,3E)-hepta-1,3-dienyl] pentanedioic acid	226.1201	4.537		*	
Flavonoid c-glycoside	Vicenin	594.1574	4.567	*	*	
Alkaloid	Benzidine	184.0998	4.809			*
Flavonoid c-glycoside	Orientin	448.0998	5.131	*	*	
Flavonoid c-glycoside	Vitexin/Isovitexin	432.1048	5.538	*	*	*
Flavonoid o-glycoside	Quercetin-3β-D-glucoside	464.0954	5.62			*
Flavonoid	Quercetin	302.0423	5.626	*	*	
Flavonoid	Kaempferol	286.0474	5.766	*	*	*
Flavonoid	Apigenin	270.0529	6.384	*	*	*
Flavonoid	3′,4′,7-Trihydroxyflavanone	272.0685	6.408			*
NA	Unknown	678.5028	6.75	*	*	*
Isoflavonoid	3-(3,4-dihydroxyphenyl) -7-hydroxy-8- [3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]chromen-4-one	432.105	6.781	*		
Chalcone	Phloretin	274.0838	6.788		*	
Steroid	5α-Androstan-3,6,17-trione	302.188	6.901		*	
Isoflavonoid	Daidzein	254.0581	7.088	*	*	*
Flavonoid	Wogonin	284.068	7.241	*		*
Flavonoid	Naringenin	272.0684	8.449	*	*	*
Fatty acid	9S,13R-12-Oxophytodienoic acid	292.2036	8.516	*		*
Sesquiterpene	Nootkatone	218.1667	8.711		*	
Volatile oil	Jasmone	164.1198	8.885		*	
Cyclic alcohol	(2R,5R,6R)-3-[(1E,3E)-hepta-1,3-dien-1-yl]-5,6-dihydroxy-2-(hydroxymethyl) cyclohexan-1-one	254.1516	8.992		*	
Flavonoid	Nobiletin	402.1307	9.422	*		
Alkaloid	1-Tetradecylamine	213.2452	10.113	*	*	*

Continued

Compound class	Putative name	MW	RT (minute)	R	M	S
Alkaloid	Bis(2-ethylhexyl) amine	241.2765	11.823	*	*	*
Triterpenoid	Ursolic acid	456.36	11.851			*
Fatty acid	9-hydroxy-2,10,10-trimethyltricyclo undec-6-ene-6-carboxylic acid	250.1567	12.462	*	*	*
Alkaloid	Octadecanamine	269.3081	12.757	*	*	*
Alkaloid	α-Pyrrolidinopropiophenone	203.1306	12.912	*	*	*
Phenolic aldehyde	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	234.1617	13.056	*	*	*
Triterpenoid	3-Hydroxylup-20(29)-en-28-oic acid	454.3444	13.49			*
Triterpenoid	18-β-Glycyrrhetinic acid	470.3393	13.572	*		*
Steroid	Testosterone undecanoate	456.36	13.766		*	
Steroid	5α-Dihydrotestosterone	290.224	14.114			*
Steroid	24,25-Epoxylanost-7-ene-3,23-diol	458.3753	14.221	*		*
Fatty acid	Hexadecanamide	255.2558	14.437	*	*	*
Fatty acid	α-Linolenic acid	278.2244	14.741	*	*	*
Cyclic ketone	2-[(5Z)-5-tetradecenyl]cyclobutanone	264.2446	14.89	*	*	*
Steroid	1-Steroyl-glycerol	358.3076	15.698	*	*	*
Amide	N,N-Diethyldodecanamide	255.2559	16.054			*
Amide	Hexadecanamide	255.2558	16.054	*		
Triterpenoid	Lupeol	426.3855	16.303	*		*
Fatty acid ester	9(Z),11(E),13(E)-Octadecatrienoic Acid methyl ester	292.2398	16.64	*		
Triterpenoid	Betulin	442.3806	16.781	*	*	*
Fatty acid ester	1,2-Cyclohexane dicarboxylic acid diisononyl ester	424.355	19.367		*	
Fatty acid	4-Phenylbutyric acid	164.0835	19.739	*	*	
Carboxylic acid	3-[hydroxy(oxido)phosphoranyl]pyruvic acid	151.98792	22.75			*
Alkaloid	Desmethyl selegiline	173.12038	22.929			*

Herbal medicine is growing in popularity year after year. As much as 60% of the world's population is said to have begun to use herbal medicine. This proportion is even higher in developing countries, reaching nearly 80% (Ahmad Khan and Ahmad, 2019). Numerous studies have indicated that *T. indica* has lipid-lowering activity and may be used to treat obesity (Aprilia *et al*., 2017; Joyeux *et al*., 1995; Lahamado *et al*., 2017). It was estimated that this activity is mediated by both antioxidant activity and inhibitory activity against proteins/enzymes involved in obesity.

However, the production of plant metabolites as well as their pharmacological activity is strongly influenced by the conditions in which the plant grows. According to the current study, the total phenolic content of the *T. indica* leaf extract grown in the rainforest zone (R) was lower (35.95% GAE) compared to the monsoon zone (M) (55.96% GAE) and the savannah zone (S) (52.30% GAE). Similarly, the total flavonoid content is highest in the S extract (2.10% QE), which is derived from tropical savannah areas that are typically dry. These results were in line with previous reports where plants that grow in areas of high ecological stress produce more metabolites than those that grow in nutrient-rich areas (Mundim and Pringle, 2018; Oni *et al*., 2013).

The antioxidant capacity of the three extracts was found to be proportional to the total phenol content (r = 0.998) rather than the total flavonoid content (r = −0.379) based on *Pearson's correlation* analysis. According to this information, it was revealed that the molecule responsible for the *T. indica* leaf extract's antioxidant activity was a nonflavonoid phenolic compound. This study differed slightly from the previous one, which demonstrated antioxidant activity was closely correlated to both total phenolic and flavonoid content (Razali *et al*., 2012). This difference was estimated to be caused by ecological factors affecting the flavonoid production in tamarind leaves during the monsoon season (M) as well as the glycosylation and methylation of flavonoids.

To unravel this ecological influence, the aqueous ethanolic extracts of three plants were analyzed using an LC-HRMS system to predict the metabolite variation, as shown in Table 2. In general, classes of molecules detected in the *T. indica* extract included alkaloids, amides, amino acids, chalcones, coumarins, fatty acids and esters, flavonoids, peptides, phenolic acids and aldehydes, sesquiterpenes, steroids, triterpenoids, and volatile oils.

Tamarind leaves are considered to be flavonoid-dense plants. The three plants contained flavonoids such as kaempferol, apigenin, naringenin, vitexin/isovitexin, and daidzein. Additionally, wogonin, 3′-4′-7-trihydroxyflavanone, and quercetin-3b-D-glycoside were found in the savannah zone (S).

Table 3. The value of K_m and V_{max} of Michaelis-Menten plot from lipase activity in the absent and present of extract. The values are presented as Mean ± SE.

Extract/ inhibitor	K_m (mM)	V_{max} (×10^{-3} mM/minute)	Lineweaver–Burk slope (K_m/V_{max})
Rainforest (R)	0.37 ± 0.18	2.08 ± 0.45	120 ± 110
Monsoon (M)	0.58 ± 0.15	2.26 ± 0.29	279 ± 39
Savanah (S)	0.21 ± 0.06*	1.55 ± 0.15*	190 ± 31
Orlistat	0.41 ± 0.23	1.71 ± 0.43	232 ± 59
DMSO	0.81 ± 0.45	3.83 ± 1.19	243 ± 71

The superscripted star symbol means significantly different to DMSO control.

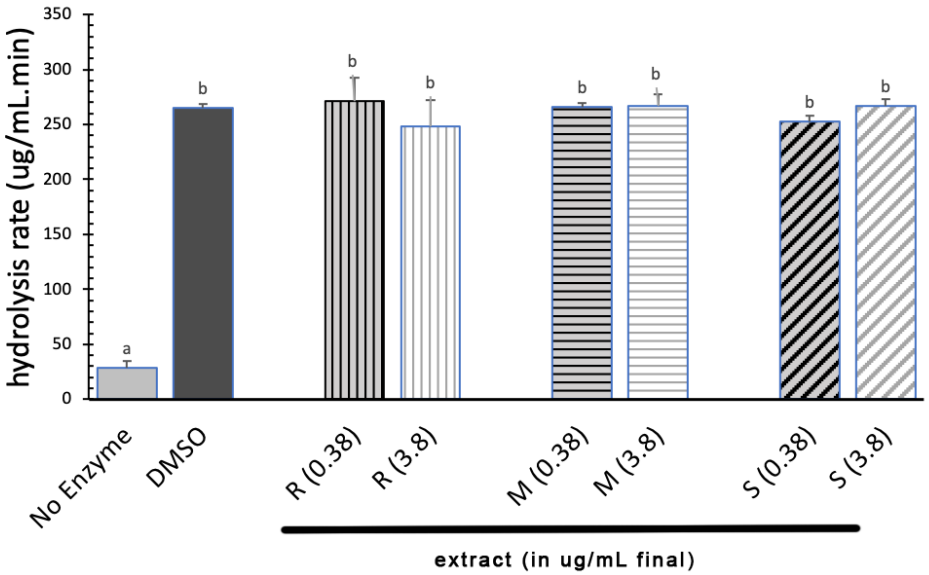

Figure 4. Rate of starch hydrolysis by pancreatic amylase in the absent and present of extract. Extract were prepared at 0.38 and 3.8 ug/ml final. (R: Rainforest, M: Monsoon, S: Savanah).

Meanwhile, nobiletin and 3-(3,4-dihydroxyphenyl)-7-hydroxy-8-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] chromen-4-one were flavonoids found exclusively in the tamarind leaves from the rainforest zone (R).

On the other hand, phytochemicals only existing in the monsoon zone (M) were 3-[(1E,3E)-hepta-1,3-dienyl] pentanedioic acid, phloretin, 5α-androstan-3,6,17-trione, nootkatone, jasmone, (2R,5R,6R)-3-[(1E,3E)-hepta-1,3-dien-1-yl]-5,6-dihydroxy-2-(hydroxymethyl) cyclohexan-1-one, and 1,2-cyclohexane dicarboxylic acid diisononyl ester. Nootkatone and jasmone were sesquiterpenes of chemical class.

Phloretin, 3′,4′,7-trihydroxyflavanone, and quercetin-3-D-glucoside were the hydroxylated compounds that were not present in the rainforest climate tamarind (R). It was possible that the absence of these components, which have a hydroxyl group at the 4' position, resulted in decreased antioxidant activity via DPPH radical scavenging. Materska (2008) reported that the hydroxyl group at position 4' on flavones is critical for their antioxidant activity. This group's antioxidant activity was said to be significantly reduced by glycosylation.

Alkaloids such as adenosine, hordenine, 1-tetradecylamine, bis(2-ethylhexyl) amine, octadecanamine, α-pyrrolidinopropiophenone, 2,2,6,6-tetramethyl-1-piperidinol, benzidine, N-methyltryptamine, and *trans*-3-indoleacrylic acid were almost evenly distributed across all climates. Alkaloids were slightly more abundant in the savannah climate (S) than in other climates. Additionally, sesquiterpenes and steroid components were found in greater abundance in tamarind monsoon climates (M), whereas terpenoids were found in greater abundance in savannah climates (S). This result corroborated previous research in which Li *et al*. (2020b) stated that ecological factors, particularly a scarcity of water, stimulated the production of sterol compounds in *Artemisia annua* and other plants. This increase was mediated by an increase in the expression of the sterol C-4 methyl oxidase (SMO1) gene, which was expressed in response to dehydration tolerance.

Pancreatic lipase and amylase inhibitory activity

Despite the fact that flavonoids were poorly absorbed in the intestine (Fakhrudin *et al*., 2019), several flavonoids have been shown to inhibit pancreatic lipase enzymes in addition to their antioxidant properties. Nonesterified flavanols like catechin, epicatechin, gallocatechin, and epigallocatechin have no effect on pancreatic lipase inhibition (Mohapatra *et al*., 2015;

Rahim *et al*., 2015). When catechins, such as epigallocatechin gallate and epigallocatechin digallate, are esterified with gallic acid, they become active in inhibiting pancreatic lipase.

The inhibitory activity of pancreatic lipase was determined using pancreatin, a digestive enzyme mixture containing lipase (6 U/mg), amylase (75 U/mg), and protease (75 U/mg) (Terra *et al*., 2016). Despite its lower activity than fresh-prepared pancreatic juice, pancreatin was acceptable to be used for food digestibility assay (Salhi *et al*., 2020).

Test results on amylase activity of all *T. indica* leaf extracts failed to inhibit pancreatic amylase at both measured concentrations ($p > 0.05$). However, as shown in Figure 3, *T. indica* leaf extract was able to significantly inhibit the pancreatic lipase enzyme ($p \leq 0.05$) at a concentration of 3.8 µg/ml (R and M extracts) compared to DMSO as solvent control using substrate concentration of 0.165 mM. This finding differed slightly from a previous report in which a 500 µg/ml final concentration of the *T. indica* leaf extract inhibited lipase by 28% less than orlistat (Abd Rahman, 2017). However, 3.8 µg/ml of the M extract was found to be more effective than orlistat in recent research. This activity was possibly influenced by the difference in its metabolites as well as growth location.

C-glycosidic flavonoids, such as vitexin, have been identified as potentially effective lipase inhibitors (Abdulai *et al*., 2021). A previous study found that kaempferol inhibited lipase via a competitive mode (Li *et al*., 2020a). Meanwhile, another study found that quercetin inhibits lipase competitively. Betulinic acid inhibited the lipase enzyme as well, but the mode of inhibition was unknown (Kim *et al*., 2012). Trendafilova *et al*. (2018) also discovered that sesquiterpene had potent lipase inhibitory activity. The mode of inhibition was poorly informed. The synergistic effect of these compounds was presumably responsible for the high lipase inhibition of the monsoon extract (M) at 3.8 µg/ml in this study. Sesquiterpenes from *Alisma orientale* were also reported to have high lipase inhibitory activity (Cang *et al*., 2017).

According to the enzyme inhibition criterion (Palmer and Bonner, 2011), the *T. indica* leaf extract inhibited pancreatic lipase on a mix-mode of inhibition. As shown in Table 3, all extracts reduced both the maximum reaction rate (V_{max}) and the Michaelis constant (K_m), as well as the slope of the Lineweaver–Burk curve. Competitive inhibition was defined as V_{max} remaining constant while K_m increased. Noncompetitive inhibition was defined as V_{max} decreasing but K_m remaining constant, while uncompetitive inhibition was defined as K_m and V_{max} changing but the Lineweaver–Burk slope remaining constant.

CONCLUSION

The lipase inhibitory activity of the *T. indica* leaf was found to be affected by its ecological environment. Among the three tropical climates, e.g., rainforest, monsoon, and savannah, *T. indica* growing in the monsoon climate inhibited lipase the most. It was thought that differences in antilipase activity were caused by differences in the types of flavonoids, alkaloids, fatty acids, and steroids. More research is needed, particularly to assess the extract's activity against other enzymes or proteins involved in the obesity mechanism, such as lipoprotein lipase, leptin, and phosphatidic acid phosphatase.

ACKNOWLEDGMENT

This research was funded by collaborative work of Research Grant of *Program Prioritas Nasional Kedeputian Ilmu Pengetahuan Teknik LIPI No. 26/A/DT/2021* and *Saintek Kemenristek 2019* scholarship program.

AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to this research, including concept and research design (TW, ENS, WRP, and AF), data acquisition (TW and AF), data analysis (AF and TW), funding (AF), manuscript drafting (TW), and critical revision of manuscript and supervision (AF, ENS, and WRP), until approval of the final version of the manuscript (ENS).

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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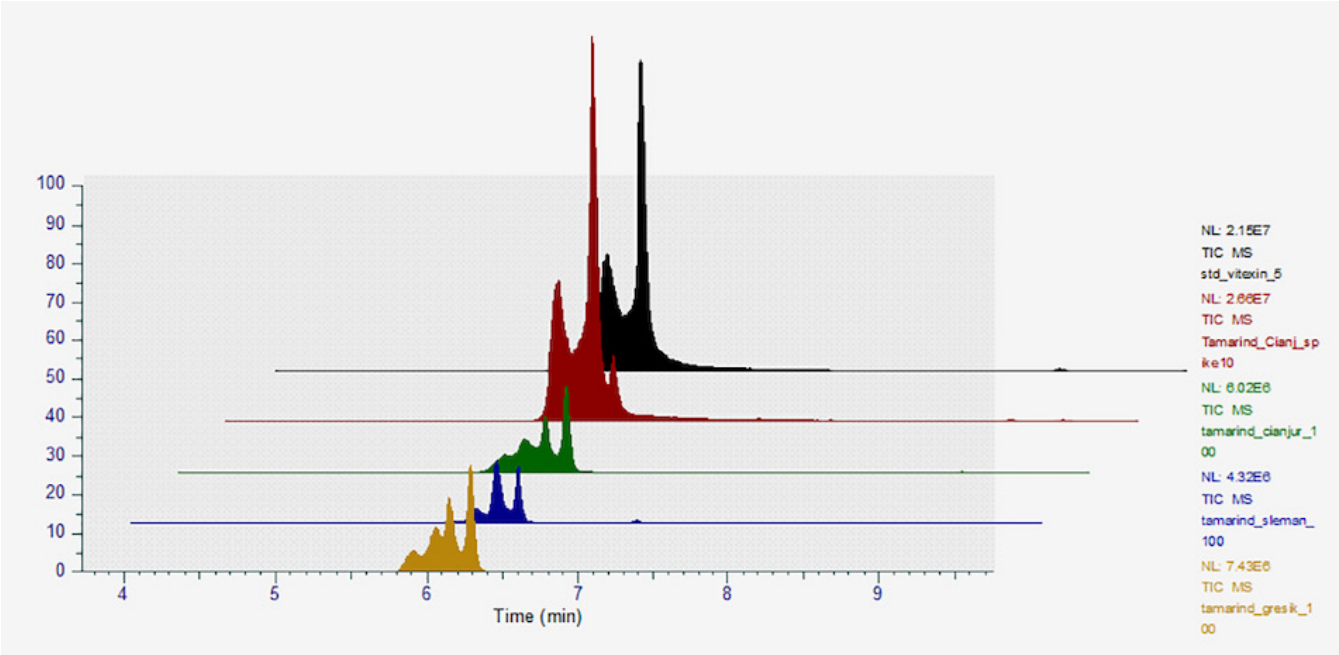
How to cite this article:
Wiyono T, Frediansyah A, Sholikhah EN, Pratiwi WR. UHPLC-ESI-MS analysis of Javanese *Tamarindus indica* leaves from various tropical zones and their beneficial properties in relation to antiobesity. J Appl Pharm Sci, 2022; 12(08):137–147.

SUPLEMENTARY MATERIAL



Suplementary Figure 1. Vitexin retention time (6 minutes) of the standard, extracts, as well as in spiked extract.