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Anti-obesity potential of selected tropical plants via pancreatic lipase inhibition

Abstract

Natural products are a vast source of potential compounds that can be developed as anti-obesity agent. One of the mechanisms of anti-obesity agents is inhibition of pancreatic lipase. Assay of 24 crude extracts for their in vitro activity against porcine pancreatic lipase (PPL) detected four extracts demonstrating high (>70%) inhibition, seven extracts had medium (30-70%) inhibition and the remaining 13 extracts exhibited low (<30%) inhibition when incubated with PPL at a concentration of 500µg/ml for 10min at 37°C. *Phyllanthus niruri* extract displayed the most potent PPL inhibitor, followed by *Orthosiphon stamineus, Murraya paniculata* and *Averrhoa bilimbi* with the IC50 value of 27.7<34.7< 41.5<55.2µg/ml, respectively. *P. niruri* & *O. stamineus* (the best two extracts) showed noncompetitive and uncompetitive inhibition, respectively. *P. niruri* & *O. stamineus* displayed total phenolic content of 431.0±0.01 and 103.0±0.01mg GAE/g dry extract, while total flavonoid content of 14.8±0.07 and 21.6±0.03mg QE/g dry extract, respectively. Both *P. niruri* & *O. stamineus* extracts showed high antioxidant activity, with EC50 values of 8.4 and 26.3µg/ml, respectively. The results suggest that *P. niruri* & *O. stamineus* may be beneficial for obesity treatment via pancreatic lipase inhibition action.

Keywords: pancreatic lipase inhibitor, *phyllanthus niruri*, *orthosiphon stamineus*, obesity

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Introduction

Pancreatic lipase inhibition is one of the approaches used to determine the potential efficacy of natural products as an anti-obesity agent. The mechanism involves inhibition of dietary triglyceride absorption, as this is the main source of excess calories. The success of orlistat as the only lipase inhibitor marketed with regulatory approval has prompted research for alternative lipase inhibitors with lesser side effects than orlistat. Up to now, many extracts and pure compounds have been identified and reported to exhibit considerable in vitro inhibitory activity against pancreatic lipase. Lipase inhibitors have been detected in different plant species, including *Ligustrum purpurascens*;[1] *Eisenia bicyclis*;[2] *Nageia wallichiana*;[3] *Panax japonicas*;[4] *Salacia reticulate*;[5] *Acanthopanax senticosus*[6] & *Nelumbo nucifera*.[7] Sharma et al.,[8]reported that 75 medicinal plants belong to different families in Korea were screened for their anti-lipase activity, using a radioactive method. According to their report, methanolic extracts of three plants namely, *Eriochloa villosa (Thunb.) Kunth*, *Orixa japonica Thunb.*, *and Setaria italica (L.) Palib*. exhibited strong in vitro anti-lipase activity with more than 80% inhibition. In China, the methanolic extracts of 37 traditional Chinese herbal medicines of different families were assayed for their in vitro activity against porcine pancreatic lipase (PPL) by using spectrophotometry with 2, 4-dinitrophenyl butyrate as a synthetic substrate. Extracts from two herbs, *Prunella vulgaris L. (Labiatae)* and *Rheum palmatum L. (Polygonaceae)*, at a concentration of 200µg/ml, significantly inhibited PPL by 74.7% and 53.8%, respectively.[9] Recently, Dechakhamphu & Wongchum[10] screened the effect of 28 medicinal plants on inhibition of pancreatic lipase and evaluate the phytochemical contents of the extracts. Their finding revealed that extracts from four herbs, *Memecylon edule Roxb.*, *Garcinia vilersiana Pierre*, *Cryptolepis elegans Wall.*, and *Phyllanthus chamaepeuce Ridl.* Strongly inhibited PPL at a concentration of 100µg/ml, by 90.97%, 92.04%, 94.64% and 95.38%, respectively. The authors also stated that there was a significant positive correlation between phenolic, flavonoid, and alkaloid contents with inhibition activity.

Obesity is recognized as a risk factor in the metabolic disorders development, hyperlipidemia, atherosclerosis, diabetes mellitus, hypertension, cancer and cardiovascular disease.[11] Pancreatic lipase inhibition is classified as one of the approaches to treat obesity due to the facts that 50-70 % total dietary fat hydrolysis were performed by pancreatic lipase.[12] Besides, pancreatic lipase inhibition does not alter any central mechanism; make it an ideal approach for obesity treatment.[13] According to the World Health Organization (WHO), medicinal plants contribute significantly to primary health care. More recently, WHO presented a Traditional Medicine Strategy for 2014-2023.[14] According to their report, the output of Chinese materia medica was estimated about US$83.1billion in 2012, an increase of more than 20% from the previous year. In the Republic of Korea, annual expenditures on traditional medicine reached US$4.4billion in 2004, rising to US$7.4billion in 2009, while out-of-pocket spending for natural products in the United States was US$14.8billion in 2008. With more people avoiding chemical drugs due to the fear of side effects, preference is now towards natural-based products, thus justifying extensive research in this field. Accordingly, the aim of this study is to examine the pancreatic lipase-inhibitory potential of 24 methanolic extracts from different parts (leaves, fruits, rhizomes, shoot and whole plant) of selected plants found in tropical countries.

Materials and methods

Materials

The plants of interest, plant parts list and source of plants are shown in Table 1. The selection of plants was based on the literature surveys of the scientific evidence and personal interview with local herbalist on the usage of selected plants for slimming treatment. The PPL (Type II) (EC 3.1.1.3), p-nitrophenyl butyrate (pNPB) and orlistat were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Table 1 List of plants selected for lipase inhibition screening

No.	Scientific name	English name	Parts tested	Source
1	*Alpinia galangal*	Galanga Root	Rhizomes	TPU, UPM
2	*Andrographis paniculata*	King of Bitter/Creat/Bile Earth	Leaves	TPU, UPM
3	*Averrhoa bilimbi*	Cucumber Tree/Tree Sorrel	Leaves	TPU, UPM
4	*Carica papaya*	Papaya	Leaves	TPU, UPM
5	*Curcuma aeruginosa* Roxb.	Nil	Rhizomes	TPU, UPM
6	*Cymbopogon nardus*	Citronella	Leaves	TPU, UPM
7	*Cymbopogon nardus*	Citronella	Shoots	TPU, UPM
8	*Garcinia atroviridis*	Nil	Fruits	Wet market, Sri Serdang
9	*Garcinia atroviridis*	Nil	Leaves	TPU, UPM
10	*Gynura procumbens* (green)	Mollucan Spinach (green)	Leaves	TPU, UPM
11	*Gynura procumbens* (red)	Mollucan Spinach (red)	Leaves	TPU, UPM
12	*Hibiscus sabdariffa*	Roselle	Fruits	Wet market, Sri Serdang
13	*Kaempferia galangal*	Fingeroot	Leaves	TPU, UPM
14	*Momordica charantia*	Bitter Gourd	Fruits	Wet market, Sri Serdang
15	*Morinda citrifolia*	Noni	Leaves	TPU, UPM
16	*Murraya paniculata*	Orange Jessamine	Leaves	TPU, UPM
17	*Orthosiphon stamineus Benth*	Cat Whiskers	Leaves	TPU, UPM; AgriPearl Sdn. Bhd.
18	*Phaleria macrocarpa*	God's Crown	Leaves	TPU, UPM
19	*Phyllanthus acidus*	Malay Gooseberry	Leaves	TPU, UPM
20	*Phyllanthus niruri*	Stone Breaker	Whole plant	TPU, UPM; AgriPearl Sdn. Bhd.
21	*Piper betle L.*	Betel	Leaves	TPU, UPM
22	*Syzygium polyanthum*	Bay	Leaves	TPU, UPM
23	*Tamarindus indica*	Tamarind	Leaves	TPU, UPM
24	*Zingiber cassumunar*	Cassumunar Ginger	Leaves	TPU, UPM

NIL, not having a common English name

TPU, UPM, taman pertanian university putra Malaysia

Methods

Method outline: Twenty-four crude extracts prepared using small-scale extraction was subjected to lipase inhibition assay screening. Four crude extracts exhibited more than 70% inhibition towards pancreatic lipase underwent IC50 and inhibition mode determination. Two crude extracts with the best IC50 value namely *P. niruri* & *O. stamineus* were chosen for further analysis. Large-scale extraction was performed followed by lipase inhibition assay, antioxidant assay and phytochemical analysis of both extracts.

Plant extractions: All plant materials (leaves, shoots, rhizomes, whole plant and fruits) were washed thoroughly with clean water. Fruits, rhizomes, and shoots were cut into small pieces, then, all plant materials were air-dried in the shade at 25-30°C. The dried plant materials were ground into fine powder using a lab-scale blender (Waring, USA). Fifty grams of the powdered materials were soaked in 500ml of 80% (v/v) methanol and incubated for three days at ambient temperature (25°C). The extracts were filtered using 150mM Whatman No. 1 filter paper and evaporated at 45°C in a rotary vacuum evaporator (Eyela, Japan). The dried crude extracts were then weighted and kept at -20°C for further investigation. In order to protect the extracts from light, the bottles containing extracts were covered with aluminium foil.

Lipase inhibition assay

Lipase preparation: The crude PPL was dissolved in 50mM phosphate buffer pH 7 (1mg/ml) and centrifuged at 12 000 x g for 5min to remove all insoluble.[15,16] Enzyme stock concentration was set at approximately 0.1mg/ml for every 1mg solid PPL powder dissolved in 1ml of buffer.

Lipase inhibition reaction: The ability of the compounds to inhibit PPL was measured using the modified method previously reported by Lewis.[16] The lipase activity was determined by measuring the hydrolysis of pNPB to p-nitrophenol at 405nm using UV-transparent 96-well plates on an ELISA reader (BIO-TEK, Synergy HT, USA) Lipase assays were performed by incubating the plant extracts (final concentration of 500µg/ml) with PPL and pNPB in reaction buffer (50mM potassium phosphate buffer, pH 7.2, 0.5% Triton X-100) for 10min. pNPB was first solubilized with 1% dimethylsulfoxide (DMSO) of the final volume, then diluted with the reaction buffer for a final concentration of 2.5mM in a 100µl reaction.

All assays were run at 37°C and reported results were the average of three replicates that were blank subtracted. Orlistat was used as a positive control. DMSO was used as a negative control and the activity was also examined with and without the inhibitor. One unit of activity was defined as the rate of reaction that produces 1µmol of p-nitrophenol permin at 37°C. Inhibition of the lipase activity was expressed as the percentage decrease in the activity when PPL was incubated with the test compounds. Lipase inhibition (%) was calculated according to the following formula:

$$Lipase\ inhibition\ (I\%) = 100 - \left[(B-b)/(A-a) \times 100\right]$$

Where A is the activity without inhibitor, a is the negative control without inhibitor, B is the activity with inhibitor and b is the negative control with inhibitor.

IC_{50} determination: The concentration of four most active plant extracts giving 50% lipase inhibition (IC_{50}) was performed using several concentrations of extracts, ranging from 0.1 to 0.6mg/ml. The IC_{50} value was calculated from the least squares regression line of the semi-logarithmic plot against percentage inhibition curves using GraphPad Prism Version 4.0 software (GraphPad Software Inc., San Diego, USA).

Inhibition mode determination: Four crude extracts exhibited more than 70% inhibition towards pancreatic lipase from screening analysis and were subjected to kinetic study in order to determine the inhibition mode. The inhibition mode was determined by Hanes-Woolf plot analysis resulting from the enzyme assay data containing increasing concentrations of pNPB (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0mM) with the absence and presence of different concentration of extracts (10 and 50µg/ml) according to the Michaelis-Menten kinetics. The Hanes-Woolf plots were designed using the GraphPad Prism Version 4.0 software (GraphPad Software Inc., San Diego, USA).

DPPH radical scavenging activity assay: Antioxidant reducing activity on 2, 2-diphenyl-1-picrylhydrazil (DPPH) was carried out according to the method of Zakaria et al.,[17] with slight modification. DPPH free radical was dissolved in methanol for the preparation of stock solution with final concentration of 120µM in reaction mixtures. *O. stamineus* and *P. niruri* extracts were prepared using methanol. Ascorbic acid (Sigma - Aldrich, USA) was used as positive control. All samples and ascorbic acid were weighted accordingly to obtain stock solution samples with initial concentration of 1000µg/ml. The percentages of the scavenging activity at this initial concentration were compared. Serial dilution was then conducted in order to obtain several different concentrations. Final concentrations that were used for the test were 500µg/ml, 250µg/ml, 125µg/ml, 62.50µg/ml, 31.25µg/ml, 15.63µg/ml, and 7.81µg/ml.

Fifty microliter of DPPH solution was added to 100µl of sample solutions in 96-well plate. The plate was then wrapped with aluminum foil to avoid exposure to light. The decrease in absorbance was determined at 515nm using a microplate reader (BIO-TEK, Synergy HT, U.S.A.) after 30min of incubation period at room temperature (28°C). The test of each sample and ascorbic acid were conducted in triplicate. Mean, standard deviation and median effective concentration (EC50) of the results obtained were determined using GraphPad Prism software (GraphPad, USA). The percentage of inhibition was calculated using the following formula:

$$\text{üüüüüüüüüüüüüü} \quad \% = 100 - \left[(\quad / \quad)\quad 100\right]$$ [18]

Where, AS=Absorbance of sample; AC=Absorbance of negative control (Methanol without sample)

Phytochemical analysis

Phytochemical analysis of *O. stamineus* and *P. niruri* extracts was carried out to quantify the total phenolic and flavonoid contents of both crude extracts.

Estimation of total phenolic content: This experiment was performed to determine the total phenolic content (TPC) according to the method described by Hakiman[19] with slight modification. Folin-Ciocalteu phenol reagent (Sigma Chemical Co., USA) was diluted with distilled water at ratio 1:1. The diluted and fresh prepared Folin-Ciocalteu reagent (20µl) was then added to 10µl of extracts in methanol (1mg/ml). Distilled water (80µl) was added to the reactions. After 3min, 40µl of 17% sodium bicarbonate (w/v) was added to the mixtures. The experiment was performed in a 96-well plate which was covered with aluminum foil. The plate was incubated and agitated at room temperature (~25°C) in the dark for 90min. Absorbance of samples was monitored at 760nm using a microplate reader (BIO-TEK, Synergy HT, USA) with distilled water served as a blank. All samples were tested in triplicate. Meanwhile, a standard curve with different concentration of gallic acid (Sigma Chemical Co., USA) (0.2, 0.4, 0.6, 0.8, and 1mg/ml) was constructed using GraphPad Prism software (San Diego, CA). The phenolic content of extracts were interpolated from the gallic acid standard curve and expressed as gallic acid equivalent per gram of dry extract (mg GAE/g dry extract).

Estimation of total flavonoid content: Total flavonoid content (TFC) was measured by the modified method of aluminum chloride ($AlCl_3$) colourimetric assay.[20,21] An aliquot of extracts (0.3ml, 1000µg/ml) was added to a 10ml test tube containing 3.4ml of 30% methanol, 0.15ml of sodium nitrite [$NaNO_2$, 5% (w/v)], and 0.15ml of $AlCl_3$ [10% (w/v)]. After 5min, 1ml of 1M sodium hydroxide (NaOH) was added. The solution was mixed and the absorbance was measured immediately against the reagent blank at 510nm using a microplate reader (BIO-TEK, Synergy HT, USA). All samples were tested in triplicate. A standard curve with varying concentrations of quercetin (20, 40, 60, 80, and 100µg/ml) was constructed using Graph Pad Prism software (San Diego, CA). The flavonoid content of extracts was interpolated from the quercetin standard curve and expressed as quercetin equivalent per gram of dry extract (mg QE/g dry extract).

Results and discussion

Lipase-inhibition of crude plant extracts

There are many techniques that can be applied to assay lipase activity either by using natural or artificial triglyceride as the substrate. These techniques include spectrophotometric, turbidimetric, titrimetric, chromogenic and immunochemical detection.[22] In this study, pancreatic lipase inhibition assay of several selected plants was successfully conducted using the spectroscopic method with pNPB as the substrate. The assay was performed using 96-well plate and was read by microplate reader. This strategy was applied to facilitate the screening step, increase robustness, and maintain reproducibility of the assay. In this study, PPL was used as a model enzyme due to its properties, which is mostly equivalent to the human pancreatic lipase (HPL) with similar enzyme kinetics and behavior.[12,23,24] Also, commercial crude PPL was available in bulk at a lower price.

The preliminary PPL inhibition assay screening detected four extracts exhibiting high (>70%) inhibition while seven extracts with medium (30-70%) inhibition and the remaining 13 plant extracts showed either low (<30%) or no inhibition when incubated with PPL at a final concentration of 500µg/ml for 10min at 37°C. All plant extracts was set at 500µg/ml as such concentration could give a consistent results with low standard deviation in the assay. Figure 1 shows the graph of the PPL inhibition assay screening result of all extracts. The four crude extracts with high inhibition towards pancreatic lipase are *O. stamineus*, *P. niruri*, *M. paniculata* and *A. bilimbi,* abbreviated as MK, DA, K and BB, respectively. All four plants with high inhibition were derived from their leaves extracts except for *P. niruri* which was prepared using the whole plant.

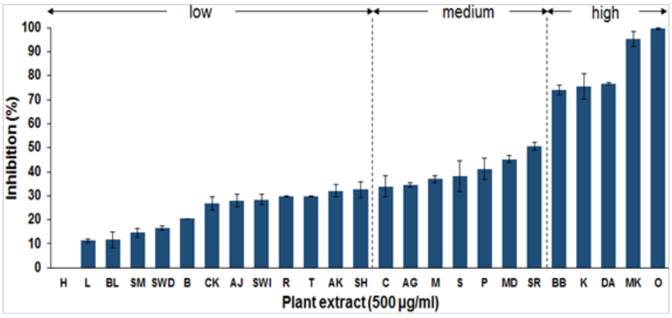

Figure 1 Screening of lipase-inhibitory activity from 24 methanolic extracts.

Plant extracts were divided into three categories, which were, low (< 30%) or no inhibition, medium (30 - 70%) inhibition and high (>70%) inhibition when incubated with PPL at a final concentration of 500µg/ml for 10min at 37°C. Values were mean±standard deviation (*n*=3). Sequence of plants from left to right (ascending order): H=*A. paniculata* (leaves), L=*A. galangal* (rhizomes), BL=*Z. cassumunar* (leaves), SM=*G. procumbens* (red) (leaves), SWD=*C. nardus* (leaves), B=*C. papaya* (leaves), CK=*K. galangal* (leaves), AJ=*T. indica* (leaves), SWI=*C. nardus* (shoots), R=*H. sabdariffa* (fruits), T=*C. aeruginosa* Roxb. (rhizomes), AK=*G. atroviridis* (fruits), SH=*G. procumbens* (green) (leaves), C=*P. acidus* (leaves), AG=*G. atroviridis* (leaves), M=*M. citrifolia* (leaves), S=*S. polyanthum* (leaves), P=*M. charantia* (fruits), MD=*P. macrocarpa* (leaves), SR=*P. betle* L. (leaves). BB=*A. bilimbi* (leaves), K=*M. paniculata* (leaves), DA=*P. niruri* (whole plant) and MK=*O. stamineus* (leaves). Orlistat served as a control and the final concentration was set at 10µg/ml.

The plant extracts with medium inhibition are known as *P. acidus* (leaves, C); *G. atroviridis* (leaves, AG); *M. citrifolia* (leaves, M); *S. polyanthum* (leaves, S); M. charantia (fruits, P); *P. macrocarpa* (leaves, MD) and *P. betle L.* (leaves, S). Meanwhile, the 13 plant extracts with low inhibition are known as *A. galangal* (rhizomes, L); *Z. cassumunar* (leaves, BL); *G. procumbens* (red) (leaves, SM); *G. procumbens* (green) (leaves, SH); *C. nardus* (leaves, SWD); *C. nardus* (shoots, SWI); *C. papaya* (leaves, B); *K. galangal* (leaves, CK); *T. indica* (leaves, AJ); *H. sabdariffa* (fruits, R); *C. aeruginosa Roxb.* (rhizomes, T); and *G. atroviridis* (fruits, AK). Among all the extracts, *A. paniculata* (leaves, H)] showed no inhibition towards PPL.

Of these 24 methanolic crude extracts, 17 were prepared from leaves, three from fruits, two from rhizomes and one each from the whole plant and shoots. Majority of extracts were prepared from the leaves, which primarily due to the significant usage of the leaves compared to other parts of the plant. The same factor contributes to the selection of rhizomes from *A. galangal* and *C. aeruginosa Roxb.*, in which the rhizomes play a significant role in traditional medication or cooking purposes.[25,26] Other factors, such as the moisture content of certain parts, especially the fruits, restricted the selection for this study since the drying process was carried out at ambient temperature (25-30°C), which could promote the growth of fungi. *M. charantia* is a common vegetable consumed by locals. The fruit is believed to be the most significant part to treat obesity or other illnesses associated with obesity.[27,28] Besides, the moisture content of *M. charantia* was low and easy to handle during drying process, while dried fruits from *G. atroviridis* and *H. sabdariffa* were easily obtained from a local market. *C. nardus* is a common ornamental in which the oil from leaves and shoots is frequently added in the slimming lotion. Both leaves and shoots are also added in the preparation of hot bath for confinement purpose.[29] Thus, crude extract of both leaves and shoots from *C. nardus* was chosen for lipase-inhibitory activity screening. In this study, *P. niruri* was prepared using the whole plant. This is due to the fact that *P. niruri* is commonly small herb and was employed in this manner in folk medicine.

The findings from IC_{50} value showed that the four most active plant extracts markedly inhibited the pancreatic lipase activity in a dose-dependent manner (Figure 2). *P. niruri* extract was the most effective pancreatic lipase inhibitor followed by *O. stamineus, M. paniculata* and *A. bilimbi* with the IC_{50} value of 27.7 < 34.7 < 41.5 < 55.2µg/ml, respectively. However, all extracts were less potent than orlistat (control) in inhibiting pancreatic lipase. Orlistat gave an IC_{50} value of 1.7µg/ml, which was about 17 times stronger than *P. niruri* crude extract. Adisakwattana et al.,[30] also reported the same when screened for lipase inhibition from selected edible plant crude extracts in Thailand.

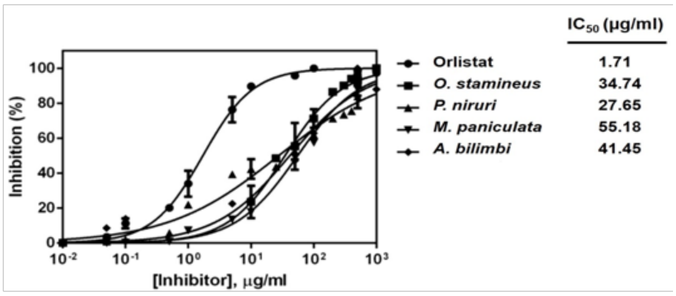

Figure 2 IC_{50} determination of selected plant extracts.

The IC_{50} value in descending order of *P. niruri<O. stamineus<M. paniculata<A. bilimbi*; 27.7<34.7<41.5<55.2µg/ml, respectively. The selection was based on the plant extracts producing the highest lipase-inhibitory activity (>70% inhibition). Orlistat served as a control. Data were presented as the mean standard deviation ($n \geq 3$).

At this point, it may be speculated that there was a presence of both active and non-active compounds in the crude extract. In the event of crude extract application, a synergistic or antagonistic action may expedite or retard the efficacy of the main compound responsible of therapeutic action.[31] The efficacy of the active compound may be affected if antagonistic action occurs. Iswantini et al.,[32] studied the pancreatic lipase inhibition of combined extracts of *Z. cassumunar*, *G. ulmifolia*, and *M. paniculata*. The results displayed that individual extract showed inhibition towards lipase, but in a very low percentage. However, when extracts of *Z. cassumunar*, *G. ulmifolia*, and *M. paniculata* of water extraction were combined with the ratio of 75:45:100ppm, the combined extracts became antagonistic towards one another, resulting in no inhibition at all. Hence, the active compound should be isolated in pure form, so that the comparison between the IC_{50} value of lipase inhibition and the inhibition mode of the chosen crude and pure extract can be understood clearly. Among the four most active plant species, *O. stamineus* and *M. paniculata* have been investigated previously for their lipase-inhibitory activity.[29,31] Iswantini et al.,[32] reported that 100ppm of *M. paniculata* aqueous extract inhibited pancreatic lipase by 25.66% which was strikingly lower than the findings in this study (75.6%) (Table 2). This might be due to different methods and solvent used for extraction. Besides, the authors used triolein, a substrate of longer chain-length to determine the pancreatic lipase-inhibitory activity in their study.

Table 2 PPL inhibition result of 24 methanolic plants extracts

Scientific name	Family	Part used	Inhibition (%) A	IC_{50} (µg/Ml)B	Yield (%) D	Abbreviation
Andrographis paniculata	*Acanthaceae*	Leaf	n	nd	16.1	H
Gynura procumbens	*Asteraceae*	Leaf	32.5+3.2	nd	5.9	SH
Gynura bicolour	*Asteraceae*	Leaf	14.6+1.9	nd	7.1	SM
Carica papaya	*Caricaceae*	Leaf	20.5+5.1	nd	11.9	B
Garcinia atroviridis	*Clusiaceae*	Fruit	32.1+2.7	nd	8.7	AK
Garcinia atroviridis	*Clusiaceae*	Leaf	34.6+1.0	nd	5.5	AG
Momordica charantia	*Cucurbitaceae*	Fruit	41.2+4.4	nd	12.6	P
Tamarindus indica	*Fabaceae*	Leaf	28.1+2.5	nd	9.5	AJ
Orthosiphon stamineus Benth	*Lamiaceae*	Leaf	95.3+3.1	34.7	9.3	MK
Hibiscus sabdariffa	*Malvaceae*	Fruit	29.6+0.2	nd	7.7	R
Syzygium polyanthum	*Myrtaceae*	Leaf	38.2+6.5	nd	11.2	S
Averrhoa bilimbi	*Oxalidaceae*	Leaf	73.9+2.0	55.2	6.5	BB
Phyllanthus acidus	*Phyllanthaceae*	Leaf	33.9+4.4	nd	5.9	C
Phyllanthus niruri	*Phyllanthaceae*	Whole plant	76.7+0.4	27. 7	19.2	DA
Piper betle L.	*Piperaceae*	Leaf	50.6+1.8	nd	12.3	SR
Cymbopogon nardus	*Poaceae*	Leaf	28.3+2.2	nd	8.8	SWD
Cymbopogon nardus	*Poaceae*	Shoot	16.6+0.8	nd	7.3	SWI
Morinda citrifolia	*Rubiaceae*	Leaf	37.0+1.4	nd	9.7	M
Murraya paniculata	*Rutaceae*	Leaf	75.6+5.4	41.5	11.5	K
Phaleria macrocarpa	*Thymelaeaceae*	Leaf	45.2+1.4	nd	19.2	MD
Kaempferia galangal	*Zingiberaceae*	Leaf	26.9+2.7	nd	7.9	CK
Zingiber cassumunar	*Zingiberaceae*	Leaf	11.7+3.3	nd	6.5	BL
Alpinia galangal	*Zingiberaceae*	Rhizome	11.4+0.7	nd	6.1	L
Curcuma aeruginosa Roxb.	*Zingiberaceae*	Rhizome	29.6+0.2	nd	9.4	T
Orlistat (Control)			99.6+0.3c	1.7	-	-

a. Percentage of inhibition represents the ability of extract to inhibit pancreatic lipase activity in the medium; concentration tested was 500µg/ml in the assay except for c. Determined based on the average of three independent replications. The values were expressed as mea±standard deviation (*n*=3).

b. IC_{50}, concentration causing 50% inhibition; concentration tested were varied from 10^{-2} to 10^{3}µg/ml in the assay.

c. Concentration tested in the assay was 10µg/ml.

d. Extraction yield of the crude extract in percentage.

N, no inhibition; nd, not determine

The results obtained from the 24 methanolic plant extracts are summarized in Table 2, including the extraction yield of all extracts. The extraction yield for all plants varied, ranging from 5.9 to 19.2%. One of the reasons was due to the physiological characteristics or different features (traits) of the plants themselves.[33] The way the plants were processed, for example; squeezing, chopping or grinding could significantly affect the yield of extract obtained, due to the density of their tissues. Grinding the plant samples into powder may provide a greater surface area and improve the extraction yield.[34] Other than that, extraction yield can be maximized by applying different solvents or manipulating different types of extraction, duration of extraction, and the washing cycle of extraction.[35,36]

In this screening study all plants, despite the different parts chosen, were extracted using 80% methanol. This is because alcohol has been proven to degrade cell walls and seeds more efficiently than water, causing more polyphenols to be released from the cells. The application of more alcohol in the solvent could reduce degradation of polyphenols by enzyme polyphenol oxidase. In addition, since almost all of the identified constituents from plants are aromatic or saturated organic compounds, the most appropriate method of choice are often obtained through ethanol or methanol extraction.[37]

Several papers have reported *O. stamineus* to be a potential anti-obesity agent. Adisakwattana et al.,[30] stated that *O. stamineus* crude extract showed strong bile acid binding activity, in which it binds glycodeoxycholic acid to a degree of 53%. Bile acid binding ability was considered as a potential way to treat hyperlipidemia.[37] It has been hypothesized as a possible mechanism of lowering plasma cholesterol level. As a result, greater amount of cholesterol is converted to bile acids to maintain a steady level in the circulation.[38] Sriplang et al.,[39] suggested that *O. stamineus* aqueous extract might be useful in the control of diabetes, one of the obesity-associated risk factor. According to their findings, *O. stamineus* extract markedly reduced hyperglycemia in streptozotocin (STZ)-induced diabetic rats, decreased plasma triglyceride and increased plasma high-density lipoprotein (HDL)-cholesterol concentrations. Yuliana[40] stated that *O. stamineus* methanolic extract has an appetite suppression effect, another mechanism of anti-obesity drug, works by reducing the desire to eat. However, no active compound responsible for the anti-obesity or anti-lipase property of *O. stamineus* and *M. paniculata* has been reported until now.

P. niruri and *A. bilimbi* have never been reported to possess anti-lipase activity so far. *P. niruri* is popular plant in folk medicine. Whole plant, fresh leaves and fruits are used in the treatment of various diseases, such as dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stone, dyspepsia, anti-hepatotoxic, anti-hepatitis-B, anti-hyperglycemic and also as antiviral and antibacterial agent.[41] It holds a reputable position in both Ayurvedic and Unani systems of medicine. Although no report on *P. niruri* having anti-lipase activity, a few reports indirectly stated the ability of this plant as anti-obesity agent. The anti-diabetic action of *P. niruri* extract has been studied by many researchers.[42–45] Okoli et al.,[44] reported the methanolic extract of aerial parts of *P. niruri* showed significant blood glucose lowering and glycemic control in diabetes. Khanna et al.,[46] reported on the effect of *P. niruri* on lipid and lipoprotein metabolism in triton-induced and cholesterol-fed hyperlipemia. According to the findings, *P. niruri* possessed lipid lowering action. Inhibition was mediated through hepatic cholesterol biosynthesis. This may support its role as a hepatoprotective agent.

A. bilimbi is medicinally used as a folk remedy for many symptoms. Various parts of *A. bilimbi* such as the leaves, bark, flowers, fruits, seeds, roots or the whole plant have been applied for medicinal purposes. The leaves are applied as a paste or poultice on itches, swellings of mumps and rheumatism, and on skin eruptions. The leaves and fruit extracts have been reported to be an effective antibacterial agent against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enteritidis*.[47] *A. bilimbi* was also reported having anti-diabetic and anti-hyperlipidaemic action, which is one of the characteristic of anti-obesity agent. Pushparaj et al.,[48] examined the hypoglycemic activity of ethanolic extract of *A. bilimbi* leaves in STZ-diabetic rats. According to their findings, they concluded that, *A. bilimbi* ethanolic leaves extract significantly lowered blood glucose by 50%, thus, has good hypoglycemic activity in STZ-diabetic rats. Ambili et al.,[49] studied the anti-hyperlipidaemic properties of *A. bilimbi* fruit using triton-induced hypercholesterolemia in rats as a model. The fruit (125mg/kg) and its water extract (50mg/kg) were found to be effective in lowering lipids in the high-fat diet fed rats. Hence, they concluded this fruit can be used as a dietary ingredient to prevent as well as to treat hyperlipidemia.

All selected plants showed inhibition towards pancreatic lipase except for *A. paniculata*. Plants with high activity may be regarded as a useful source of anti-obesity agents. Conversely, plants with medium to low inhibition did not seem to play a significant role as potential anti-lipase agent and can be regarded as poor inhibitors. Traditionally, *A. paniculata* was used to treat high blood pressure, jaundice, diabetes, fever, skin problems, flu, respiratory disease, and act as anti-venom against snakebite in East and Southeast Asia.[50–53] As the prevalence of obesity and diabetes is common in our society, research on plants with anti-lipase, anti-diabetic, and anti-hyperlipidemic action has great value in modern therapeutics. The facts show that all of the four most active plant extracts, which are *P. niruri*, *O. stamineus*, *M. paniculata* and *A. bilimbi*, can be considered potent herbs with anti-obesity properties for future research, as evidenced by the findings.

Inhibition mode

The inhibition mode of the four most active plant extracts has been visualized using graphical representation of the Michaelis-Menten equation, Hanes-Woolf plot; [S]/v versus [S], as shown in Figure 3. The inhibition mode was plotted from the enzyme assay data containing increasing concentrations of pNPB substrate (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0mM) with the absence and presence of difference concentration of extracts (10 and 50µg/ml). It is important to calculate Km (substrate concentration at which the reaction rate is half-maximum or half of Vmax or also known as Michaelis-Menten constant) and V_{max} (maximum rate at saturating substrate concentrations) to understand the enzyme characteristics.

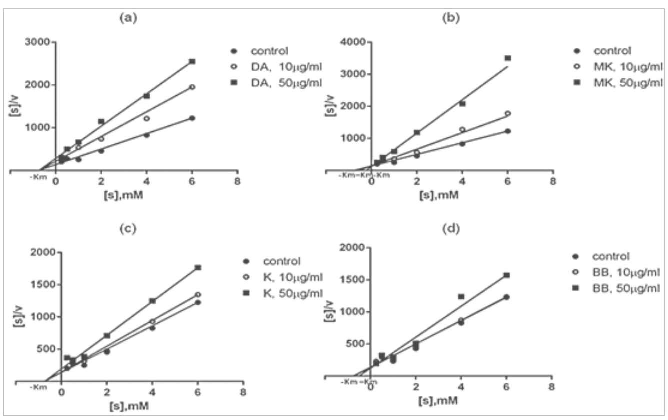

Figure 3 Inhibition mode determination.

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Hanes-Woolf plot; [S]/v versus [S] of kinetic analysis for PPL at two different concentrations of (a) *P. niruri* extract (Abbreviated as DA), (b) *O. stamineus* extract (Abbreviated as MK), (c) *M. paniculata* extract (Abbreviated as K) and (d) *A. bilimbi* extract (Abbreviated as BB). Data were presented as the mean standard deviation ($n \geq 3$).

The enzyme kinetics result showed that *P. niruri* and *M. paniculata* extracts exerted inhibitory effect on pancreatic lipase in a noncompetitive manner. As depicted from the graph, when the extracts' concentrations were increased, the values for the y-intercept in the equation for each curve increased, whereas the x-intercepts remained at a fixed point showing that these inhibitors do not affect Km but the Vmax decreased. Km value for PPL was 0.76mM and V_{max} was 0.0057mM/min. Kinetic study in the presence of *P. niruri* and *M. paniculata* extracts showed reduction of Vmax to 0.0027 and 0.0036mM/min, respectively. Therefore, it was concluded that both *P. niruri* and *M. paniculata* extracts inhibited pancreatic lipase by binding with the free enzyme or the enzyme-substrate complex.

O. stamineus and *A. bilimbi* showed uncompetitive inhibition towards pancreatic lipase activity. The uncompetitive inhibition lines intersected on the y-axis whereas the value for the x-intercept in the equation for each curve increased; illustrating that such inhibitors affected both Vmax and Km. Km value for PPL was 0.76mM and Vmax was 0.0057mM/min. In the presence of *O. stamineus* and *A. bilimbi* extracts, Km was reduced to 0.23 and 0.51mM, respectively, while V_{max} was reduced to 0.0019 and 0.004mM/min. Therefore, both *O. stamineus* and *A. bilimbi* extracts inhibited pancreatic lipase by binding with only the enzyme-substrate complex. This is due to the fact that uncompetitive inhibition takes place when an enzyme inhibitor binds only to the complex formed between the enzyme and the substrate. This shows that the substrate binding could cause a conformational change to take place in the enzyme that enables the inhibitor to bind to the enzyme-substrate complex.[54]

These findings may present a preliminary result of the pancreatic lipase inhibition mode of these four potential plants. However, it should be kept inmind that the extracts tested are in crude form. The presence of both active and non-active compounds in the crude extract may create synergistic action. Furthermore, there is a possibility that these extracts are having more than one lipase inhibitor. Thus, it might affect the results. Accordingly, it is essential to check the inhibition mode of the pure compound later to validate the verdicts. However, a few papers have reported the same kinetic study in the presence of crude extracts.[55–57]

Antioxidant activity of *O. stamineus* and *P. niruri* extracts

Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions.[58] Antioxidants can also protect the human body from free radicals and reactive oxygen species effects. They retard the progress of many chronic diseases as well as lipid peroxidation.[59] Natural antioxidants tend to be safer and are known to exhibit a wide range of biological effects including anti-lipase, antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic and vasodilatory activities.[59,60]

The potential of *O. stamineus* and *P. niruri* extracts as free radical scavenger was analysed using DPPH scavenging assay. This non-enzymatic assay is widely accepted as a tool to assess free radical scavenging activity of antioxidant for its advantage of economical and ease.[61] DPPH is a stable nitrogen-centered organic radical with a characteristic absorption within 515 - 528nm.[62] This easy and convenient assay is based on the reduction of the alcoholic DPPH solution in the presence of antioxidant. Antioxidants interrupt free radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end product, diphenylpicrylhydrazine (DPPH), which does not initiate or propagate further oxidation of lipids. This causes the purple-coloured DPPH to lose its chromophore and turn it into a yellowish product (α,α-diphenyl-β-picryl hydrazine).[63–65]

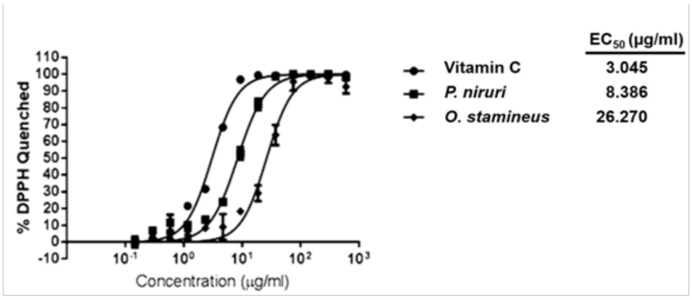

Figure 4 Antioxidant activities of *O. stamineus* and *P. niruri* extracts at different concentration.

A transform of dose-response curve of DPPH radicals scavenging activities of the *P. niruri and O. stamineus* crude extracts was presented in Figure 4. The transform of dose-response curve was constructed to determine the EC_{50} value of both extracts. EC_{50} value refers to the effective concentration of antioxidant agent that is required to scavenge 50% of the radicals under experimental condition. The lowest EC_{50} value indicates the strongest ability of sample to act as DPPH radical scavenger. It was observed that the DPPH radical-scavenging activity increased as the concentration of the extract increased. The scavenging activity of *O. stamineus* and *P. niruri* extracts toward DPPH radicals increased from 0.2 to 500µg/ml and was 91.8% and 93.6% at a concentration of 500µg/ml, respectively.

As shown in Figure 4, *P. niruri* extract has reached maximum plateau within the range of concentration. *P. niruri* extract showed greater ability to inhibit DPPH radical than *O. stamineus* extract with recorded EC_{50} value of 8.386µg/ml. Although *O. stamineus* extract displayed lower antioxidant activity when compare with *P. niruri* extract, with an EC_{50} value of 26.27µg/ml, *O. stamineus* extract may be classified as a good antioxidant agent. Ascorbic acid (Vitamin C) served as the positive control with an EC_{50} of 3.045µg/ml.

The antioxidant activities were defined as inhibition percentage of DPPH in DPPH assay. Vitamin C served as the positive control. Data were presented as the mean standard deviation (n=3).

TPC and TFC of *O. stamineus* and *P. niruri* extracts

Phenolic acids and flavonoids are secondary metabolites widely distributed in the plant kingdom. Plant phenolics and flavonoids are very effective free-radical scavengers and their antioxidant activities are well documented.[66] Hence, plants which contain high levels of phenolics and flavonoids are indeed a good source of antioxidants and therefore it is important to quantify the total phenolics and total flavonoids in plant extracts as they might have some beneficial effects on health.[67,68]

The amount of phenolic compounds in *P. niruri and O. stamineus* extracts were determined using the Folin-Ciocalteau colourimetric method. By manipulating the regression equation of gallic acid calibration curve (y=2.640x+0.1759, r2=0.9878), the TPC of each extract was calculated and expressed as gallic acid equivalent (GAE). TPC of *O. stamineus* extract was 103+0.08mg GAE/g dry extract while *P. niruri* extract displayed dramatically higher TPC with 431+0.01mg GAE/g dry extract (Table 3). This indicates that *P. niruri* has good potential as a source for natural antioxidant to prevent free radical oxidative damage. The antioxidant activity of phenolic compounds is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen. In addition, they may also possess metal chelation properties.[69] Levels of flavonoid compounds in *O. stamineus* and *P. niruri* extracts were determined using the aluminium chloride colourimetric assay. By manipulating the regression equation of quercetin calibration curve (y=0.00669x - 0.004667, r2=0.9977), the TFC of each extract was calculated and expressed as quercetin equivalent (QE). The TFC of *O. stamineus* extract was slightly higher than *P. niruri* extract with 21.6±0.03 and 14.8±0.07mg QE/g dry extract, respectively (Table 3).

Table 3 Summary of TPC, TFC, anti-lipase activity and antioxidant activity of *P. niruri* and *O. stamineus* extract

	Total Phenolic Content (Mg GAE/G Dry Extract)	Total Flavonoid Content (Mg QE/G Dry Extract)	Anti-Lipase Activity [I C_{50} (µg/Ml)]	Antioxidant Activity [E C_{50} (µg/Ml)]
P. niruri	431.0±0.01	14.8±0.07	28.13	8.39
O. stamineus	103.0±0.08	21.6±0.03	44.05	26.27

The values were expressed as mean±standard deviation (*n*=3)

O. stamineus contains several chemically active constituents, but one of the most important classes of compounds is the phenolic group.[70] A study of 50% ethanol extract of *O. stamineus* leaves for its anti-tumor potential showed an ability to inhibit colon tumor in mice could be attributed mainly to its antioxidant-rich polyphenolic contents, the caffeic acid derivatives, polymethoxylated flavonoids and terpenes; particularly rosmarinic acid, eupatorin, sinensetin, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, and betulinic acid.[71] The authors found that flavonoids and phenolics in the extract possessed very promising antiangiogenic properties. Akowuah et al.,[66] studied the phytochemicals content of *O. stamineus* collected from different locations in Malaysia. They reported the samples from various locations showed a remarkable degree of variation of their antioxidant activity. Besides, the TPC of the methanol extracts varied from 6.69mg caffeic acid/g dry weight in the sample from Pasir Puteh (Kelantan) to 10.20mg caffeic acid/g dry weight in the sample from Parit (Perak). Variations in the free-radical activity may be due to agronomic practices and environmental conditions which at once could affect both soil fertility levels and phytochemicals content.[66]

Extensive research on *P. niruri* active constituents and their pharmacological activities was begun in the mid-1960s. Several classes of chemicals have been found in *P. niruri*, including lignans, alkaloids, benzenoids, coumarins, lipid, sterol, tannin, terpenes, saponins and flavonoids.[72] A few flavonoid from *P. niruri* that have been reported so far include quercetin, rutin, astragalin, quercitrin, isoquercitrin, kaempferol-4'-rhamnopyranoside, fisetin-4-o-glucoside and nirurin.[73,74] Harish & Shivanandappa[72] investigated the antioxidant activity and hepatoprotective potential of *P. niruri*. Their findings also revealed the high potency of the crude extracts of *P. niruri* in free radical scavenging, inhibition of reactive oxygen species and lipid peroxidation in both *in vivo* and *in vitro*. However, they stated that there was no correlation between antioxidant activity and phenolics content of the *P. niruri* extracts, which suggests that besides phenolics, other chemical constituents may contribute to antioxidant activity. This, at once suggests that although individual phenolics may have considerable antioxidant potential, there could be synergistic or antagonistic interactions between phenolic and non-phenolic compounds. Khanna et al.,[46] studied the lipid-lowering activity of *P. niruri* in triton and cholesterol fed hyperlipidemic rats, however, they did not stated the active phytochemical constituent that responsible for the lipid lowering activity. Up to now, there is still a lack of information on the relationship between anti-lipase activity and their phenolic or flavonoid compounds on a large scale to reveal whether their relationship is positive or negative. Overall, the results suggest that *P. niruri* & *O. stamineus* may be beneficial for obesity treatment via pancreatic lipase inhibition action.

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Conflict of interest

The author declares no conflict of interest.

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