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Research Article

Lipid and Blood Pressure Lowering Effects of *Mikania micrantha* Through Enzymatic Inhibition

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ABSTRACT

Mikania micrantha Kunth (Asteraceae) is a plant traditionally used to reduce the risk of hyperlipidemia and hypertension. There is limited information on the anti-hyperlipidemic and anti-hypertensive effects of the various *M. micrantha* leaves and stem extracts. This study aimed to examine the *in vitro* potential of different parts of *M. micrantha* (leaves and stem) extracts in inhibiting hyperlipidemia-related enzymes, *i.e.*, pancreatic lipase (PL), lipoprotein lipase (LPL) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), in addition to the hypertension-related, *i.e.*, angiotensin-I converting enzyme (ACE). This study spectrophotometrically determined the inhibitory activities of hot water, cold water, 70% ethanol, and ethyl acetate *M. micrantha* leaves and stem extracts against the above-said enzymes using PL, LPL, HMGR, and ACE inhibition assays. The study found that the ethanol stem (ETS) extract exhibited the highest PL inhibitory activity (IC_{50}=4.49±2.50 µg/mL), albeit the difference was insignificant ($p > 0.05$) compared to orlistat (IC_{50}=0.31±0.01 µg/mL). Meanwhile, the ethanol leaves (ETL) extract yielded the highest LPL (IC_{50}=1.42±0.48 µg/mL) and HMGR inhibitory activity (50.12±3.44%), although the greatest ACE inhibition was observed for the hot water stem (HWS) extract (97.47±1.19%). However, the result was insignificant ($p > 0.05$) compared to other extracts and captopril (98.42±0.93%). In brief, the extracts generally exhibited remarkable inhibitory activity against PL, LPL, HMGR, and ACE, thus conveying the *M. micrantha* extracts' anti-hyperlipidemic and anti-hypertensive potentials.

Keywords: Angiotensin-converting enzyme, HMG-CoA reductase, Hypertension, Lipase inhibition, Mikania micrantha, Pancreatic lipase

Introduction

The importance of plants to human life can be seen in their diverse utilization in medicine and food as nutraceuticals. Plants are a large source of new bioactive molecules with therapeutic potential. They have increasingly become attractive alternatives to prevent or reduce cardiovascular disease (CVD) risk factors such as hyperlipidemia and hypertension [1]. Since ancient times, medicinal plants have been identified to play a vital role in new medicinal substances and drug development. *Mikania micrantha* Kunth is a fast-growing perennial climber plant from South and North America, which also grows in Southeast Asia countries such as Malaysia and Indonesia [2, 3]. *M. micrantha* receives recognition in various fields of study, particularly for ethnopharmacology and therapeutic application. The plant extracts have been reported to exhibit antioxidant [4], antibacterial [3], anti-microbial [5], anti-proliferative [6], anti-dermatophytic [7], and anti-cancer activities [8, 9]. The bioactivities described above are related to the presence of terpenoids in the plant,

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such as sesquiterpene lactones, flavonoids, alkaloids, steroids, reducing sugars, saponins, phenolics, and tannins [10-12].

Hyperlipidemia and hypertension are the main contributing risk factors to developing cardiovascular diseases. Hyperlipidemia refers to elevated cholesterol, triglycerides, or both, while hypertension is characterized as constant elevation of systolic (> 140 mmHg) and diastolic (> 80 mmHg) blood pressures [13]. Different strategies are used to treat and manage hyperlipidemia and hypertension and among them, targets lowering the serum LDL-C levels for hypercholesterolemia (by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase) [14]. This consequently prevents angiotensin II formation, leading to hypertension (by inhibiting the angiotensin-I converting enzyme) [15]. Other strategies include inhibiting pancreatic lipase (PL) and lipoprotein lipase (LPL), the key enzymes in lipid metabolism responsible for hydrolysing dietary fats in the intestine and the lipolysis of triglycerides in lipoprotein, respectively [16]. Thus, inhibiting the actions of both PL and LPL can help reduce dietary triglyceride absorption and decrease dietary cholesterol absorption.

There are reports of established lipases, HMG-CoA reductase, and ACE inhibitors such as orlistat, statins, and captopril. However, these oral medications have certain limitations and side effects [17-19]. Therefore, the quest for more effective and safer drugs must continue for the benefit of humankind. Another crucial factor to consider is the choice of extraction solvents, which affects the plant extracts' chemical composition and biological activity [20]. There is growing interest in replacing modern medicine with traditional plant extracts and some natural components in plants for treating hyperlipidemia and hypertension. In fact, plant-based products are generally considered less toxic compared to synthetic counterparts [21]. Hence, this study aimed to determine the inhibitory activity of *M. micrantha* extracts on PL, LPL, HMG-CoA reductase, and ACE inhibitors which are the enzymes responsible for hyperlipidemia and hypertension.

Material and Methods

Sample Preparation and Extraction

Mikania micrantha leaves and stems were collected and identified (the voucher sample; SBID 051/15) at the Forest Research Institute Malaysia (FRIM), Kepong, Selangor, Malaysia. The leaves and stems were separated, washed, oven-dried at 35°C for 72 h, ground to a fine powder, and stored at -20°C until further use. *M. micrantha* leaves and stems were extracted using hot water, cold water, 70% ethanol, and ethyl acetate [4].

Pancreatic Lipase (PL) Inhibition Assay

The PL activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenylbutyrate (*p*-NPB) as the substrate [22]. Crude porcine pancreatic lipase (PPL) (5 mg/mL) was suspended in MOPS-EDTA buffer (10 mM, pH 6.8 with 1 mM EDTA) and centrifuged at 1800 g for 10 min to recover the clear supernatant containing the enzyme. The enzyme solution (6 µL), 20 µL of the extracts or orlistat (0 - 100 µg/mL), and 169 µL Tris-HCl buffer (100 mM Tris and 5 mM $CaCl_2$, pH 7.0) were mixed, and pre-incubated for 15 min at 37°C. Finally, 5 µL of *p*-NPB solution (10 mM in acetonitrile) was added and incubated at 37°C for 60 min, before the absorbance was read on a microplate reader at 405 nm.

Lipoprotein Lipase (LPL) Inhibition Assay

LPL from bovine milk (Sigma, EC 3.1.1.34) was freshly prepared and diluted to 25 U/mL by suspending in 100 mM cold sodium phosphate buffer (containing 150 mM sodium chloride and 0.5% (v/v) Triton X-100, pH 7.2 at 37°C). A mixture of the LPL solution (6 µL), 20 µL of the extracts or orlistat (0 - 100 µg/mL), and 169 µL of Tris-HCl buffer (0.02 M, pH 8.0) were pre-incubated at 37°C for 15 min. Then, 5 µL of the substrate solution (10 mM *p*-nitrophenylbutyrate in acetonitrile) was added to the mixture and incubated for 60 min at 37°C. The release of *p*-nitrophenol was measured at 405 nm using a microplate reader [23].

HMG-CoA Reductase (HMGR) Inhibition Assay

Briefly, a 1 µL aliquot of *M. micrantha* extracts (1 mg/mL) and positive controls (simvastatin and pravastatin, 100 µM) were pipetted into the 96-well plate, followed by the addition of 100 mM potassium phosphate buffer (pH 7.4, contains 120 mM potassium chloride, 1 mM EDTA, and 5 mM dithiothreitol), 2 µL NADPH, 6 µL of HMG-CoA substrate (400 µM) and 1 µL of HMG-CoA reductase. The mixture was incubated at 37°C for 10 min, and the rate of NADPH consumed was

measured at 340 nm using a microplate reader [24].

Angiotensin-I Converting Enzyme (ACE) Inhibition Assay

ACE from rabbit lung (Sigma, EC 3.4.15.1) was suspended in sodium borate buffer (0.1 M, pH 8.3 with 0.3 M NaCl) to give a final concentration of 4 mU/mL. Briefly, a mixture containing 5 µL of the ACE solution, 5 µL of extracts/captopril (1 mg/mL), 25 µL of 0.1 M sodium borate buffer and 25 µL of 5 mM hippuryl-histidyl-leucine (HHL) were interspersed in a microcentrifuge tube, before incubation at 37°C for 60 min. Then, 35 µL of 1 M HCl was added, followed by 70 µL of pyridine and 35 µL of benzenesulphonylchloride (BSC). The mixture was cooled on ice for 15 min, and 200 µL of the solution was transferred into the 96-well plate and read using a microplate reader at 410 nm [25].

Statistical Analysis

All results were expressed as mean ± standard error of three independent experiments' mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) using IBM SPSS Statistics (version 22). Finally, the IC_{50} values were estimated by the GraphPad Prism software, version 7 (San Diego, CA, USA).

Results and Discussion

Research on natural plant-based inhibitors to combat hyperlipidemia and hypertension is increasing due to the adverse effects of commercial drugs, for instance, statins, whose side effects include distal muscle weakness, headache, acute renal failure and pancreatitis [26]. ACE inhibitors cause hyperkalemia, fatigue, nausea and renal impairment [27]. Fruits, vegetables, and medicinal plants are the preferred alternative due to their safety, affordability, and availability for long-term consumption [28]. While numerous reports show that *M. micrantha* juice (by boiling hot water) could reduce cholesterol, high blood pressure, and glucose [2], scientific data are lacking to prove this traditional use. Notably, this study highlights the inhibitory capacity of the *M. micrantha* leaves and stem extracts against PL, LPL, HMG-CoA reductase, and angiotensin-I converting enzymes. Inhibition of these key enzymes is one of the common mechanisms used for the management of hyperlipidemia and hypertension.

Pancreatic Lipase (PL) Inhibitory Activity

Pancreatic lipase (PL) assay by spectrophotometry method is one of the simplest methods for evaluating PL activity. PL is the enzyme responsible for the hydrolysis of dietary triglycerides, yielding monoglycerides and fatty acids [29]. In this assay, the PL activity was estimated from the hydrolysis of *p*-nitrophenylbutyrate (*p*-NPB) to *p*-nitrophenol at 405 nm. In contrast, the inhibitory activity (I%) was calculated using the formula: $1-[(B-b) / (A-a)] \times 100$ [22]. Porcine pancreatic lipase was selected as the test enzyme since it represents 85% homology to the human enzyme and possessed similar enzyme kinetics and behaviour [30].

As shown in Table 1, the PL inhibition gave IC_{50} values between 4.56 ± 0.07 to 28.97 ± 4.22 µg/mL and 4.49 ± 2.50 to 42.37 ± 4.63 µg/mL, for the leaves and stem extracts, respectively. It is germane to indicate here that a low IC_{50} value indicates the highest PL inhibitory activity. The study found that the ethanol stem- (IC_{50}; 4.49 ± 2.50 µg/mL) followed by hot water leaves- (IC_{50}; 4.56 ± 0.07 µg/mL) and ethanol leaves (IC_{50}; 8.02 ± 1.56 µg/mL) extracts showed the greatest PL inhibition, although the differences between the extracts were insignificant ($p > 0.05$). The extracts also did not exhibit a significant difference ($p > 0.05$) when compared to orlistat (IC_{50}; 0.31 ± 0.01 µg/mL) (Table 1). Similarly, the different parts of the plant (leaves and stem) were not significantly different ($p > 0.05$) for the tested extracts, except for the hot water *M. micrantha* leaves extract, which showed the highest PL inhibition (Table 1).

To prevent or treat hyperlipidemia, inhibiting pancreatic lipase (PL) could hinder cholesterol uptake via the intestine. Since the extraction of phytochemicals from plant samples hinges closely on the solvent choice, this study used solvents of different polarities to determine the best PL inhibitor from the *M. micrantha* leaves and stem extracts. The present study discovered that *M. micrantha* extracted using 70% ethanol and ethyl acetate demonstrated higher PL inhibitory activity than other solvents (Table 1).

The outcome here proves that these organic solvent-derived plant extracts (ethanol and ethyl acetate) exhibited greater potential in inhibiting pancreatic lipase than the aqueous extracts. The

Table 1. IC_{50} values for pancreatic lipase and lipoprotein lipase inhibitory activity of *M. micrantha* extracts

Extraction solvents	Pancreatic Lipase (IC_{50} (µg/mL)		Lipoprotein Lipase (IC_{50} (µg/mL)	
	Leaves	Stems	Leaves	Stems
Hot water	4.56 ± 0.07^{ab}	42.37 ± 4.63^{d}	4.59 ± 0.87^{a}	8.04 ± 2.75^{a}
Cold water	28.97 ± 4.22^{cd}	16.93 ± 1.99^{bc}	2.34 ± 1.88^{a}	2.70 ± 1.79^{a}
70% ethanol	8.02 ± 1.56^{ab}	4.49 ± 2.50^{ab}	1.42 ± 0.48^{a}	4.26 ± 1.23^{a}
Ethyl acetate	18.00 ± 3.78^{bc}	16.73 ± 0.70^{bc}	7.35 ± 2.68^{a}	5.69 ± 2.46^{a}
Orlistat	0.31 ± 0.01^{a}		1.98 ± 1.22^{a}	

Note: Data are expressed as means ± SEM. Means with different letters within and between groups for pancreatic lipase and lipoprotein lipase are significant at $p < 0.05$. Orlistat is a positive control. The concentration of extracts and orlistat used was 0 – 100 µg/mL. The lowest IC_{50} indicates the highest inhibitory activity [33].

study findings are supported by earlier observations showing alkaloids, carotenoids, glycosides, polyphenols, polysaccharides, saponins, and terpenoids in medicinal plants as potential inhibitors of PL [31]. Likewise, Khatun *et al*. [32] reported the presence of alkaloids, terpenoids, tannins, and cardiac glycosides in the *M. micrantha* 70% ethanol extracts [30]. The same was expected for alkaloids, tannins, cardiac glycosides, and terpenoids found in the ethyl acetate extracts of *M. micrantha* such as caryophyllene, germacrene D, and thujopsene, which are promising PL inhibitors [4].

Lipoprotein Lipase (PL) Inhibitory Activity

Similar to the PL inhibition assay, lipoprotein lipase (LPL) inhibitory activity of *M. micrantha* extracts was determined by estimating the amount of *p*-nitrophenylbutyrate (*p*-NPB) hydrolysed to *p*-nitrophenol at 405 nm by LPL. Results revealed the LPL inhibition gave IC_{50} values between 1.42 ± 0.48 to 7.35 ± 2.68 µg/mL and 2.70 ± 1.79 to 8.04 ± 2.75 µg/mL, for the leaves and stem extracts, respectively (Table 1). The ethanol leaves (ETL) extract demonstrated the greatest LPL inhibitory activity (IC_{50}; 1.42 ± 0.48 µg/mL), although the value was insignificantly different ($p > 0.05$) when compared against all plant extracts with orlistat. The extracts of the different plant parts also markedly inhibited the LPL activity, despite their potency being insignificantly different ($p > 0.05$) (Table 1).

PLP is the enzyme responsible for the triglycerides' hydrolysis in the triglycerides-rich lipoprotein, yielding free fatty acids and glycerol. It is the rate-limiting enzyme for plasma triglyceride clearance and tissue uptake of fatty acids [34]. The study found that the *M. micrantha* extracts were similar in inhibiting LPL (Table 1), substantiating the reportedly positive correlation of flavonoids with LPL inhibitory activity [35]. Hence, the flavonoids in *M. micrantha* such as astragalin, quercetin, luteolin, kaempferol, baicalein, quercetin-3-*O*-rutinoside, and luteoloside might have imperative role in inhibiting the LPL [36]. In fact, flavonoids have been positively correlated with LPL inhibitory activity [35], plausibly attributed to the compounds' synergistic effects in the sample [37].

Orlistat works locally in the lumen of the stomach and small intestine by forming a covalent bond with the serine active site in PL [18]. Since orlistat is minimally absorbed and works only within the intestine, it cannot inhibit the actively functioning LPL in the vascular lumen of blood from performing its function [23]. Therefore, PL and LPL different sites of action might explain the better-performing orlistat in inhibiting the PL than LPL. Further studies are needed to evaluate the bioavailability of *M. micrantha* extracts *in vivo* to confirm their efficacy.

HMG-CoA Reductase (HMGR) Inhibition Activity

In this study, HMGR activity was determined spectrophotometrically by estimating the decrease in absorbance at 340 nm, representing the oxidation of NADPH by the catalytic subunit HMGR in the presence of HMG-CoA as the substrate [38]. Notably, HMGR, the rate-limiting enzyme in the endogenous cholesterol biosynthesis pathway, catalyses the two-step reduction of HMG-CoA to mevalonate, with NADPH as the cofactor [39].

The study shows that the percentage inhibition of HMGR activity was between 40.55 ± 0.34 to 50.12 ± 3.44% and 16.13 ± 0.89 to 43.29 ± 5.97%, for the *M. micrantha* leaves and stem extracts, respectively (Figure 1). The ethanol leaves (ETL) showed the highest HMGR inhibition (50.12 ±

3.44%) among the different tested extracts comparatively but was not significantly different from others ($p > 0.05$), except for the hot water stem extract (HWS, 16.13 ± 0.89%) ($p < 0.05$). The positive controls, simvastatin (85.66 ± 0.88%) and pravastatin (72.83 ± 0.98%), exhibited greater HMGR inhibitory activity than all other *M. micrantha* extracts ($p < 0.05$). The findings seen here are consistent with the reported simvastatin showing 89.2 ± 3.5% inhibition of HMGR [40]. Statins are structurally similar to the HMG-CoA and could inhibit HMGR activity by competing with the substrate for the enzyme's active site [41].

The inhibition of HMG-CoA reductase has been correlated to many plant extracts' fundamental mechanism of cholesterol-lowering action. Various methanolic extracts of medicinal plants show anti-HMGR activity with percentage inhibition ranging from 2.8% to 74.1% [40]. Plants from the Asteraceae family, such as *Cosmos caudatus* (Ulam raja) and *Eclipta prostrate* (urang-aring) exhibited 13.2% and 24.4% inhibition of HMGR, respectively [40]. This shows the promising potential of *M. micrantha* extracts as a cholesterol-lowering agent following the > 30% *in vitro* inhibiting potential of HMGR activity. The *M. micrantha* extract is a promising cholesterol-lowering agent as it exhibits *in vitro* inhibition activity of HMGR. Another recent *in vivo* study on the effect of ethyl acetate stem extract of *M. micrantha* demonstrated the extract's anti-hypercholesterolemic properties, which improved the lipid profile, enzyme inhibitory, lipid peroxidation reduction, and lipid accumulation in hypercholesterolemia-induced rats [42].

Luteolin and stigmasterol (a phytosterol analogous to cholesterol in mammals) present in *M. micrantha* are promising HMGR inhibitory compounds [43-45]. Luteolin was found to possess an anti-hyperlipidemic effect *in vivo* due to its ability to reduce the total cholesterol, triglycerides, and free fatty acid in the isoproterenol-induced myocardial infarction of male Wistar rats [45]. The interaction of plant extracts with the enzyme-substrate complex could alter the enzyme's active site, thus preventing cholesterol formation. This could be the mechanism of anti-HMG-CoA reductase of *M. micrantha* extracts, potentially reducing cholesterol synthesis at the mevalonate pathway.

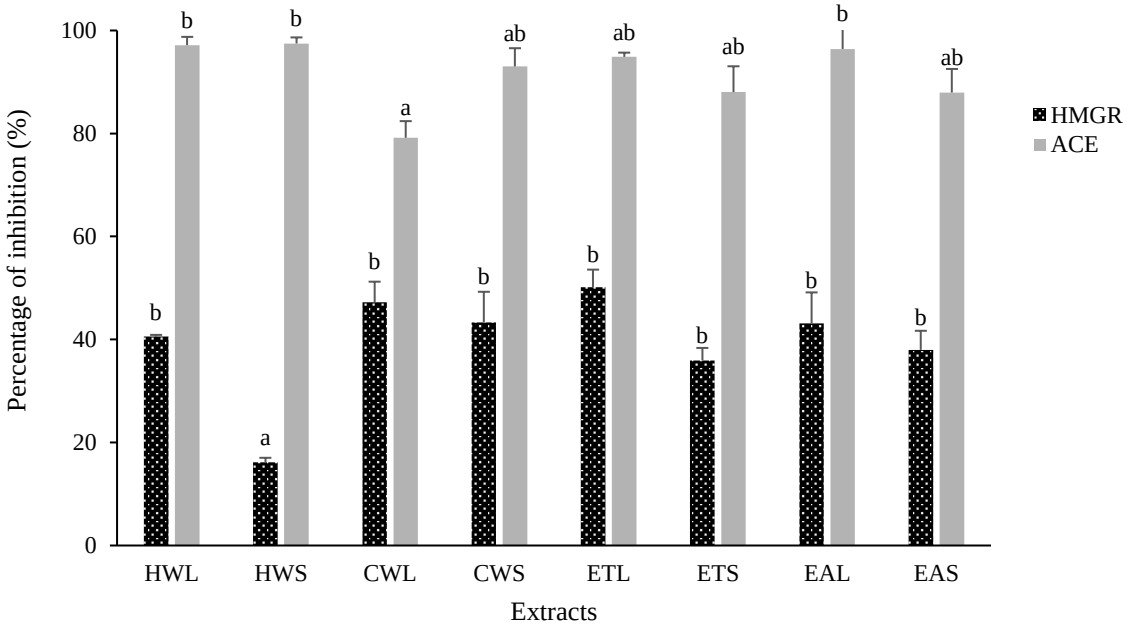

Figure 1. HMGR and ACE inhibitory activity of *M. micrantha* extracts. Bars with different letters between extracts of the same enzyme inhibitory activity are significant ($p < 0.05$). HWL, hot water leaves; HWS, hot water stems; CWL, cold water leaves; CWS, cold water stems; ETL, ethanol leaves; ETS, ethanol stems; EAL, ethyl acetate leaves; EAS, ethyl acetate stems. The concentration of extracts used was 1000 µg/mL.

Angiotensin-I Converting Enzyme (ACE) Inhibition Activity

The angiotensin-I converting enzyme plays an important role in regulating hypertension by converting angiotensin I to angiotensin II, which increases blood pressure. Hypertension can be managed using inhibitors that block the ACE activity [46].

The results of ACE inhibition activity are illustrated in Figure 1. The percentage inhibition of ACE activity ranged between 79.17 ± 3.23% to 97.15 ± 1.61% and 87.95 ± 4.58% to 97.47 ± 1.19%, for the *M. micrantha* leaves and stem extracts, respectively (Figure 1). The HWS extract showed the greatest ACE inhibition (97.47 ± 1.19%), but the difference was insignificant ($p > 0.05$) between the plant extracts and captopril (98.42 ± 0.93%), except for cold water leaves extract. In addition, the *M. micrantha* leaves and stem extract insignificantly inhibited ACE ($p > 0.05$) when compared to each other (Figure 1).

A previous study reported captopril inhibited ACE with an IC_{50} of 4 µg/mL [25], 0.015 µg/mL [27], and 0.02 µM [47]. Notably, the varying IC_{50} values for captopril seen here might be due to the difference in the test conditions, the captopril concentration, and the origin of the ACE enzyme. Other factors include the substrate used, measurement method (i.e., spectrophotometric, fluorometric, HPLC, electrophoresis, radiochemistry), and even the calculation methods. Hence, a direct comparison of the present study with other researchers is difficult to achieve.

The inhibition of ACE by medicinal plant extracts to reduce hypertension was previously reported for the 80% methanolic extracts of six plants, which showed > 50% ACE inhibition at a concentration of 330 µg/mL [48]. Also, the results from this study were comparable to the polyphenolic-rich fractions of Davidson's plum. This native Australian fruit showed inhibited ACE at 91.3% for the same tested concentration (1 mg/mL) [49]. Another polyphenolic-rich fraction of methanol extracts of native Australian herbs demonstrated ACE inhibition <30% at 1 mg/mL concentration [50]. This indicates the potential of *M. micrantha* extracts as a potent ACE inhibitor, which activity is comparable to captopril and exhibited greater than 79% ACE inhibition at 1 mg/mL.

M. micrantha is rich in terpenoids, phenolics, flavonoids, alkaloids, tannins, saponins, glycosides, and steroids [51,52]. The literature consistently show that natural ACE inhibitors can be found in plant secondary metabolites containing terpenoids, phenolic, flavonoids, hydrolysable tannins, xanthones, procyanidins, and caffeolyquinic acid derivatives [44]. In addition, Loizzo *et al*. demonstrated that flavonoids inhibit ACE activity, thus implying that flavonoids are an outstanding source of functional anti-hypertensive agents [47]. Hence, terpenoids such as α-caryophyllene, germacrene D, and thujopsene isolated from the ethyl acetate extracts of *M. micrantha* from our previous study [4], could be the potential ACE inhibitors, as reported by Adefegha et al. [53].

Notably, secondary metabolites reportedly partake in two potential ACE inhibition activity mechanisms: First, their interaction with the zinc ion on the enzyme active site. Secondly, interactions of the metabolites by forming hydrogen bonds between the hydroxyl groups and amino acids in the enzyme active site, which block the ACE's catalytic activity [54]. Despite the activity of a single compound, the maximum ACE inhibitory activity in the *M. micrantha* extracts was likely due to the synergistic activity of various compounds [55]. Figure 2 illustrates the potential schematic mechanisms of *M. micrantha*, the potential as an anti-hyperlipidemic and anti-hypertensive agent for *in vitro* management of hyperlipidemia and hypertension. In short, this study demonstrated the promising use of the three *M. micrantha* extracts to inhibit PL, LPL, HMGR, and ACE.

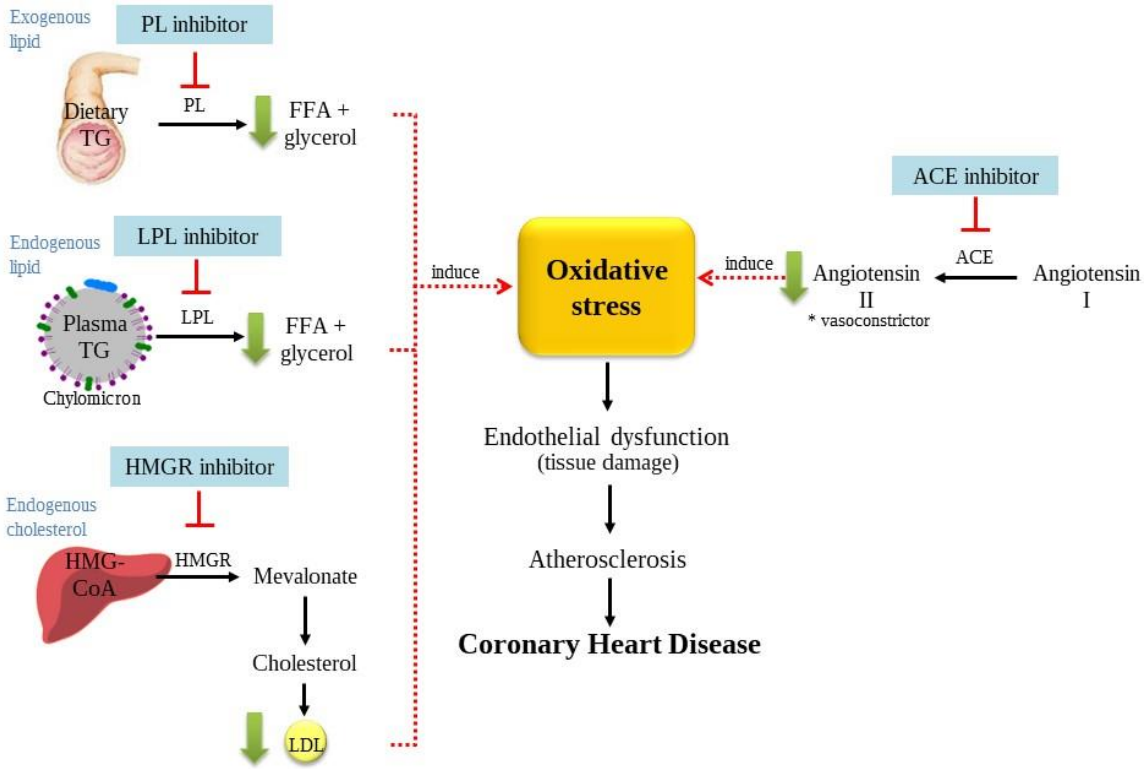

Figure 2. Schematic mechanism of action of *M. micrantha* as anti-hyperlipidemic and anti-hypertensive agents. Pancreatic lipase (PL), lipoprotein lipase (LPL), HMG-CoA reductase (HMGR) and angiotensin-I converting enzyme (ACE) are the enzymes responsible for hyperlipidemia and hypertension. Hyperlipidemia and hypertension are the contributing factors that lead to atherosclerosis and consequently to coronary heart disease. Common therapeutic approaches are directed at lowering the intestinal triglycerides (TG) and lipoprotein, endogenous cholesterol formation, and angiotensin II formation via inhibition of lipases (PL and LPL), HMGR, and ACE, respectively.

Conclusion

In short, *M. micrantha* extracts exhibited remarkable potential inhibitory activities against PL, LPL, HMGR, and ACE. This study's results can guide further investigation of the potential use of *M. micrantha* in the prevention and treatment of oxidative-damaged related diseases such as hyperlipidemia and hypertension.

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