

In vitro inhibitory activity of *Annona squamosa* leaves against enzymes associated with metabolic disorders

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ABSTRACT: *Annona squamosa* L. is a popular medicinal plant of Bangladesh having several traditional uses. To give the scientific clarification for ethnomedicinal uses of this plant, this study was aimed to analyze the phytochemical profile of methanol extract of *Annona squamosa* leaves (designated as MEAL) and evaluate its inhibitory properties against α -amylase, pancreatic lipase, and angiotensin converting enzyme (ACE). Different *in vitro* methods were used to perform enzyme inhibition assays and kinetic studies for MEAL and its phytochemical profile was analyzed by gas chromatography-mass spectroscopy (GC-MS). In enzyme inhibition assays, MEAL showed significant ACE inhibitory activity whereas it had moderate inhibition against pancreatic lipase. The IC_{50} of MEAL was found to be $9.26 \pm 0.71 \mu\text{g/mL}$ and $6.90 \pm 0.58 \mu\text{g/mL}$ for ACE and lipase enzymes, respectively. MEAL was also found as mixed type inhibitor of ACE in enzyme kinetic study. In case of α -amylase, MEAL (IC_{50} : $608.8 \pm 7.02 \mu\text{g/mL}$) showed poor inhibition activity when compared with standard drug acarbose (IC_{50} : $28.53 \pm 1.35 \mu\text{g/mL}$). In addition, presence of some compounds in MEAL identified by GC-MS, were also consistent with these enzyme inhibitory activities. Thus, this study proves the enzyme inhibitory abilities of *Annona squamosa* leaves for the first time and it might be a potential tool for the treatment of hypertension and obesity.

KEYWORDS: *Annona squamosa*; leaves; inhibition; α -Amylase; lipase; ACE.

1. INTRODUCTION

Obesity, diabetes mellitus and hypertension are the most common metabolic diseases in the world. The prevalence of these diseases has been increased tremendously in the past few decades [1,2]. There is a close association between cardiovascular diseases, obesity and diabetes. The major basis for this link is the obesity associated resistance of available insulin in the cells and the destruction of insulin secretory mechanisms [3]. Insulin resistance is considered as one of the major pathogenesis of type 2 diabetes (T2D) and some other pathophysiological conditions such as hyperlipidemia and hypertension [3]. One of the therapeutic approaches for treating obesity, diabetes mellitus and hypertension is inhibition of pancreatic lipase, α -amylase and angiotensin-converting enzyme (ACE) activities. Alpha-amylase and pancreatic lipase catalyze the hydrolysis of complex oligosaccharides and triacylglycerols in the digestive tract. So, inhibition of these enzymes reduces postprandial blood glucose and lipid levels [4]. In addition, inhibition of the angiotensin-converting enzyme (ACE) is one of the important strategies to control hypertension because this enzyme catalyzes the conversion of angiotensin I into a vasoconstrictor peptide known as angiotensin II [5]. Although several inhibitors of these enzymes such as orlistat, acarbose and captopril are used as established drugs to treat obesity, type-2 diabetes and hypertension, the chronic use of these synthetic inhibitors may be associated with many undesirable side effects [6-8]. In this regard, it is a demand of time to find new effective inhibitors against α -amylase, pancreatic lipase and angiotensin-converting enzyme (ACE) from natural sources having minimal side effects [9]. Several previous studies showed that plant extracts can inhibit the activity of these enzymes [10-12]. So, screening on plant sources for inhibitor of these enzymes can be a sound approach to control these metabolic diseases.

Annona squamosa L. is such an evergreen plant that belongs to the Annonaceae family and it is mainly located in tropical and subtropical regions including Bangladesh [13]. All parts of *Annona squamosa* with a

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long history of traditional practices, are used to treat different diseases such as heal boils, ulcers, heart diseases, cancer etc. [14-16]. In recent decades, several studies have confirmed that this plant contained bioactive compounds of different categories like acetogenin, alkaloids, steroids, terpenoids, saponins, and phenolics, which exert various biological activities [13, 17-18]. Moreover, methanolic extract of *Annona squamosa* leaves exhibited potent free radical scavenging activity and contained rich quantity of phenolic compounds [19]. Previous information described that plant extracts with rich phenolic content and antioxidant activity showed inhibitory effect against enzymes related to metabolic disorders [20-22]. Having concern with these findings, this study was designed to evaluate the inhibitory properties of *Annona squamosa* leaves against α -amylase, pancreatic lipase and ACE along with analyzing the phytochemical profile by GC-MS.

2. RESULTS AND DISCUSSION

2.1. Pancreatic lipase, α -amylase and ACE inhibitory activity of MEAL

Diabetes mellitus is a metabolic disorder which manifest clinically as elevated blood glucose level. Treatment approach that maintains normal level of glucose in the blood, is most effective for diabetes mellitus [23]. In case of non-insulin dependent type II diabetes, increased breakdown of starch by α -amylase is considered as the early stage. So, the inhibition of α -amylase can significantly decrease the postprandial increase in blood glucose level after taking of a mixed carbohydrate diet. In this study, MEAL showed poor activity and its IC_{50} value was found to be $608.8 \pm 7.02 \mu\text{g/mL}$ (Table 1) whereas acarbose (a reference inhibitor) exhibited a stronger inhibitory activity against α -amylase (IC_{50} : $28.53 \pm 1.35 \mu\text{g/mL}$) (Table 1). Previous reports of *in vitro* and *vivo* experiments demonstrated the antidiabetic and α -glucosidase inhibitory properties of water, n-hexane, ethanol and methanol extracts of *Annona squamosa* leaves and methanolic extract was found to be less active in respect to other extracts [24-27]. So, the results of this study were consistent with previous findings. In case of pancreatic lipase inhibition, MEAL showed a dose dependent inhibitory effect on lipase and its IC_{50} value was found to be $6.90 \pm 0.58 \mu\text{g/mL}$ (Table 1). On the other hand, orlistat (a reference inhibitor) displayed a stronger inhibitory activity against lipase (IC_{50} : $0.94 \pm 0.04 \mu\text{g/mL}$). The pattern of lipase inhibition by MEAL had some similarity with the previous findings where the pancreatic lipase inhibitory activity of *Cornus mas* and *Cornus alba* was better than orlistat [28].

In type-2 diabetes mellitus, damaging of the small blood vessels occurs in human body which causes the walls of the blood vessels to stiffen. This leads to develop hypertension which is a risk factor of cardiovascular diseases. Angiotensin converting enzyme (ACE) catalyzes the formation of angiotensin II (a physiologically potent vasoconstrictor) by cleavage of angiotensin 1 and so inhibition of ACE has been considered as one of the appropriate therapeutic approaches for regulating the blood pressure [29]. In this investigation, MEAL also showed potent inhibition against ACE (IC_{50} : $9.26 \pm 0.71 \mu\text{g/mL}$) as compared with captopril, a standard ACE inhibitor (IC_{50} of $6.38 \pm 0.42 \mu\text{g/mL}$) (Table 1). Thus, the inhibition of ACE activity by MEAL revealed that leaves of *Annona squamosa* could possess the ability to control blood pressure by reducing the production of angiotensin II.

Table 1. IC_{50} values of MEAL and corresponding standard inhibitor against pancreatic lipase, α -amylase and angiotensin converting enzyme (ACE)

Enzyme	MEAL	Orlistat	Acarbose	Captopril
$IC_{50} (\mu\text{g/mL})$				
Lipase	6.90 ± 0.58^a	0.94 ± 0.04^b	-	-
α -Amylase	608.8 ± 7.02^a	-	28.53 ± 1.35^b	-
ACE	9.26 ± 0.71^a	-	-	6.38 ± 0.42^b

Results are means \pm standard deviations (SD) of triplicate determinations. Along the same row, values having different superscript letters vary significantly ($P < 0.05$). IC_{50} : concentration of extract that inhibited enzyme activity by 50%

2.2. Kinetic analyses of ACE inhibition

Michaelis-Menten kinetic studies were used to elucidate the mechanism of inhibition of ACE by MEAL. These kinetic analyses of ACE inhibition were accomplished using several concentrations of the substrate (HHL), in the presence of MEAL and the data are illustrated in the Lineweaver-Burk (Fig. 1). From

these plots, the K_m value for ACE was calculated to be 5.15 and 6.17 mM at the concentration of 100 and 200 $\mu\text{g/mL}$, respectively. In case of inhibition of ACE by MEAL, the Lineweaver–Burk plots (Fig. 1) displayed no intersection on the y or x axis. The plots cross to the left of the $1/V$ axis but above the $1/S$ axis which indicates that MEAL showed a mixed-type mode (both competitive and non-competitive) of inhibition with respect to the substrate (HHL). This mixed-type inhibition suggests that the MEAL affected the affinity of the enzyme for the substrate (HHL) but did not bind at the active site [30]. Previous study has shown that *Limonium michelsonii* exhibited same type of inhibition mode against ACE [31].

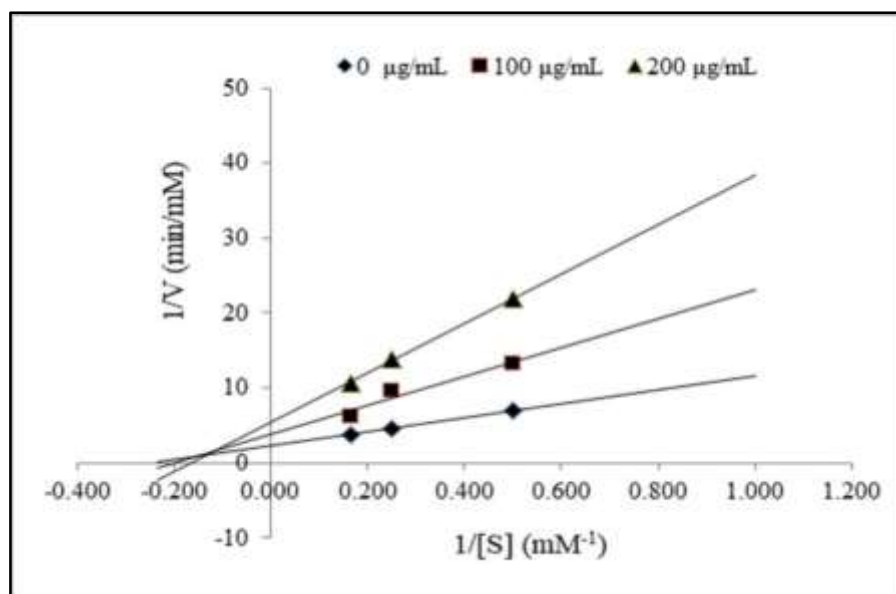


Figure 1. Lineweaver-Burk plots for the inhibition of ACE by MEAL

2.3. Chemical composition analysis of MEAL by GC-MS

Gas chromatography-mass spectroscopy (GC-MS) is a combined analytical technique used to determine and identify compounds present in a plant sample. GC-MS plays an essential role in the phytochemical analysis and chemotaxonomic studies of medicinal plants containing biologically active components [32]. Phytochemical screening of methanolic extract of *A. squamosa* leaves (MEAL) confirmed the presence of some therapeutically significant phytochemicals. As shown in GC-MS chromatogram (Fig. 2) and table 2, the identified components represent 94.0% of the total extract. The major constituents were 2-pentanone, 4-hydroxy-4-methyl- (2.07%), p-xylene (5.89%), o-xylene (6.26%), triethylene glycol monododecyl ether (33.66%), benzoic acid, 3,4,5-trihydroxy- (0.90%), longifolene (0.92%), bicycloelemene (1.36%), β -bourbonene (0.86%), caryophyllene (2.06%), germacrene-D (8.56%), 9,12,15-octadecatrienoic acid, ethyl ester (2.46%), luteolin (5.86%), 5,8,11-eicosatriynoic acid, methyl ester (5.25%), glaucine (5.25%) and palmitone (10.06%).

Previous studies have demonstrated that flavonoids are an excellent source of functional antihypertensive products. In this study, the remarkable ACE inhibition by MEAL may be explained by the existence of a flavonoid, luteolin (Fig. 3) since it exhibited an inhibitory effect against angiotensin-converting enzyme (ACE) activity [33]. In addition, the presence of gallic acid (i.e., benzoic acid, 3,4,5-trihydroxy-) (Fig. 3) is also a contributor in the inhibition of ACE by MEAL because gallic acid has shown ACE inhibition in previous investigation [34-35]. Moreover, moderate inhibition of pancreatic lipase by MEAL was also found here and according to the earlier reports, this observed inhibitory property may be due to the presence of hexadecanoic acid methyl ester and longifolene (Fig. 3) [36-38]. Previously longifolene with preferable binding energy and good inhibition constant, showed potential lipase inhibitory activity [38]. So, this compound is the active ingredient responsible for the lipase inhibitory activity of MEAL. Thus, some of the identified compounds play their key role in the inhibition of enzymes by MEAL.

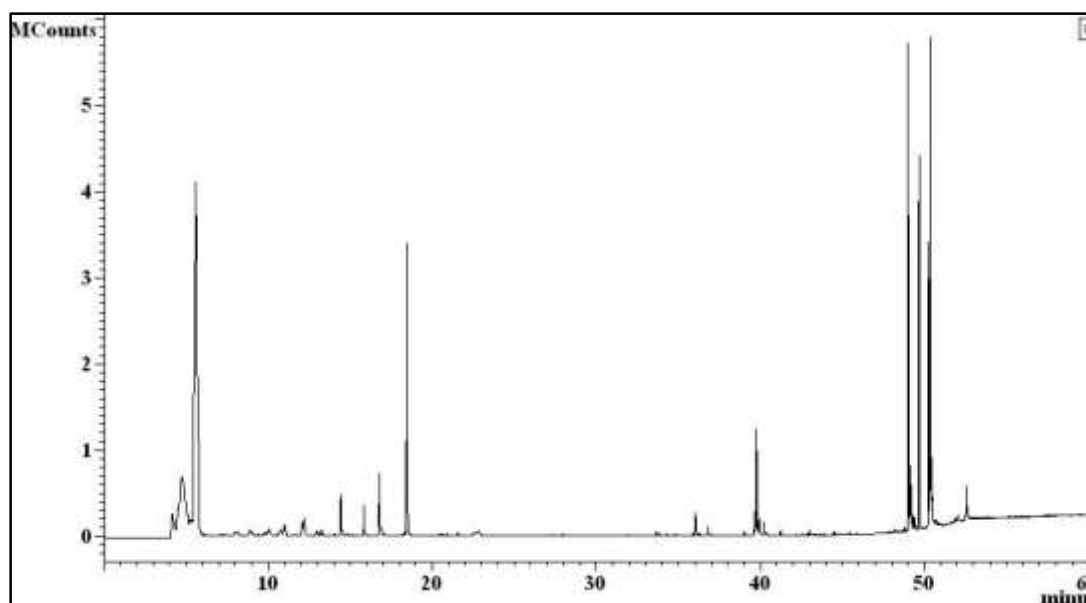


Figure 2. GC-MS chromatogram of MELA

Table 2. Chemical composition of MELA analyzed by GC-MS.

No.	RT	Identified compound	Formula	MW	Area (%)
1	4.16	2-Pentanone, 4-hydroxy-4-methyl-	C ₆ H ₁₂ O ₂	116.08	2.07
2	4.72	p-Xylene	C ₈ H ₁₀	106.16	5.89
3	4.75	o-Xylene	C ₈ H ₁₀	106.16	6.26
4	5.59	Triethylene glycol monododecyl ether	C ₁₈ H ₃₈ O ₄	318.27	33.66
5	8.027	Benzoic acid, 3,4,5-trihydroxy-	C ₇ H ₆ O ₅	170.12	0.90
6	8.89	Isopropylbenzene	C ₉ H ₁₂	120.09	0.50
7	10.75	Benzene, 1-methyl-3-(1-methylethyl)-	C ₁₀ H ₁₄	134.10	0.53
8	10.97	Benzene, 2-ethyl-1,4-dimethyl-	C ₁₀ H ₁₄	134.10	0.73
9	12.17	Longifolene	C ₁₅ H ₂₄	204.35	0.92
10	14.43	Bicycloelemene	C ₁₅ H ₂₄	204.35	1.36
11	15.84	β-bourbonene	C ₁₅ H ₂₄	204.35	0.86
12	16.90	Caryophyllene	C ₁₅ H ₂₄	204.35	2.06
13	18.57	Germacrene-D	C ₁₅ H ₂₄	204.35	8.56
14	36.05	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	0.62
15	39.65	9,12-Octadecadienoic acid, methyl ester	C ₁₈ H ₃₂ O ₂	280.24	0.40
16	39.75	9,12,15-Octadecatrienoic acid, ethyl ester	C ₂₀ H ₃₄ O ₂	306.25	2.46
17	49.00	Luteolin	C ₁₅ H ₁₀	286.23	5.86
18	49.67	5,8,11-Eicosatrienoic acid, methyl ester	C ₂₁ H ₃₀ O ₂	314.22	5.25
19	50.25	Glaucine	C ₂₁ H ₂₆ NO ₄	356.41	5.25
20	50.34	Palmitone	C ₃₁ H ₆₂ O	450.82	10.06

Abbreviation: MF, molecular formula; MW, molecular weight; RT, retention time.

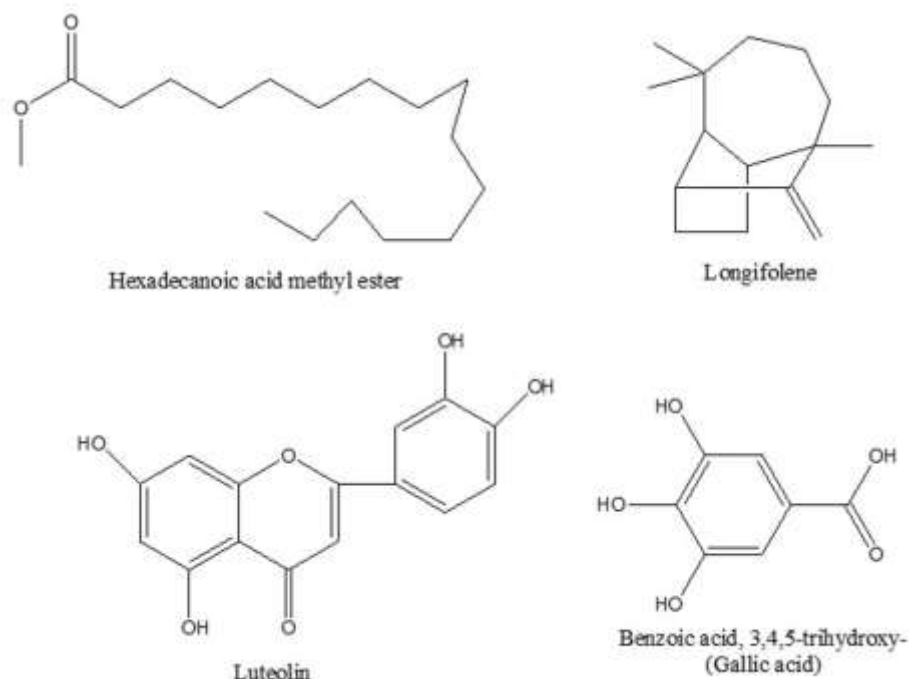


Figure 3. The chemical structure of active compounds identified in MELA by GC-MS

3. CONCLUSION

The overall findings of this study demonstrated the remarkable inhibitory activity of methanolic extract of *Annona squamosa* leaves (MELA) against angiotensin converting enzyme (ACE) and its mode of inhibition was found to be mixed type. MELA also exhibited moderate inhibitory properties against pancreatic lipase. The GC-MS analysis of MELA confirmed the presence of bioactive compounds like luteolin, gallic acid, hexadecanoic acid methyl ester and longifolene which have anti-hypertensive and lipase inhibitory properties. Hence, this study validates the scientific and traditional use of *Annona squamosa* leaves in the management of hypertension and obesity. However, additional studies on humans and experimental animals will be useful to elucidate the *in vivo* efficacy of *Annona squamosa* leaves.

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

Porcine pancreatic lipase, α -amylase, p-nitrophenyl palmitate (p-NPP), angiotensin I-converting enzyme (ACE), pyridine, acarbose, orlistat and captopril were purchased from Merck (Darmstadt, Germany) whereas hippuryl-histidyl-leucine (HHL), BSC (benzene sulphonyl chloride), were collected from Dojindo EU GmbH, Germany. Methanol, dimethyl sulfoxide (DMSO) and other solvents of HPLC grade were purchased from Labscan (Thailand)

4.2. Sample collection and authentication

Leaves of *Annona squamosa* L. were collected from the area near the Rajshahi University Campus and the collection period of plant materials was March, 2021. This plant was authenticated by Professor Dr. A. H. M. Mahbubur Rahman (a taxonomist), Department of Botany, University of Rajshahi. After authentication it was deposited in the herbarium of Botany department under the specimen record number of 1048. The collected leaves were cleaned and dried at room temperature. Then dried samples were ground to a fine powder by a grinder machine and stored in air tight glass containers for further use.

4.3. Plant extract preparation

The leaf powder (150 g) was soaked in 450 mL methanol and it was kept at room temperature for 72 hrs. Then the extract was filtered through a filter paper and concentrated using a rotary evaporator. 2.7 g residue was obtained as methanolic extract of *Annona squamosa* leaves (designated as MEAL) and it was stored at -80 °C for the various analyses.

4.4. Pancreatic lipase inhibition assay

The inhibitory activity of MEAL was evaluated against pancreatic lipase using a reported method with minor modification where p-nitrophenyl palmitate (p-NPP) was used as a substrate [39]. The enzyme under the reaction conditions hydrolyses p-NPP to release p-nitrophenol, a color agent that can be monitored at 410 nm. In this inhibition assay, extract and orlistat of different concentrations were used and these were prepared in DMSO. Lipase (15 mg) was dissolved in Tris-buffer (50 mM, pH 8) and then it was stirred for 15 min and centrifuged at 2000 rpm for 10 min. The clear supernatant was recovered and used as lipase solution. In a test tube, 1 mL sample (extract or orlistat) was mixed with 0.5 mL lipase solution. After incubation for 30 min at 37 °C, 1 mL substrate p-NPP (3 mM in 2-propanol) was added into it. This reaction mixture was incubated for 2 h at 37 °C and finally the absorbance of this mixture was recorded at 410 nm against a blank. The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition of lipase} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} and A_{sample} are the absorbance of control and sample, respectively. The control contained all constituents except a test sample. Orlistat was used as a positive control. All the tests were done in triplicate and the mean values were used to draw graph from which the IC_{50} values ($\mu\text{g/mL}$) were determined.

4.5. Alpha-amylase inhibition assay

The α -amylase inhibitory activity of MEAL and acarbose (used as positive control) was conducted by following the previous protocol with minor modification [40]. First 250 μL of sample and 125 μL of α -amylase solution (45 units/mL) in 0.02 M sodium phosphate buffer (pH 6.9) was mixed and incubated at 25 °C for 10 min. After incubation, 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 6 mM NaCl) was added to each tube. The reaction mixtures were further incubated at 25 °C for 10 min. Then the reaction was stopped with 250 μL of 3M HCl and subsequently 150 μL of a mixture solution of 5 mM I_2 and 5 mM potassium iodide was added to each test tube as color developing reagent. The absorbance of each test tube was measured at 620 nm and the α -amylase inhibitory activity was calculated as follows:

$$\% \text{ Inhibition of } \alpha\text{-amylase} = [(A_{\text{cont}} - A_{\text{samp}}) / A_{\text{cont}}] \times 100$$

Where A_{samp} was defined as absorbance of the sample and A_{cont} was absorbance of the control that included all other reagents and the enzyme with the exception of the test sample.

4.6. Angiotensin 1-converting enzyme (ACE) inhibition assay

There are several methods to determine the ACE inhibitory activity of plant extracts. Here the method described by Khan and Kumar (2019), was utilized where hippuryl-histidyl-leucine (HHL) was used as substrate [41]. The assay mixture contained 125 μL of 0.05 M sodium borate buffer pH 8.2 containing 0.3 M NaCl, 50 μL of 5 mM HHL and 25 μL of ACE enzyme solution (2.5 mU) which was pre-incubated with different sample concentrations of test sample (MEAL and captopril). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 0.2 mL of 1M HCl. Pyridine (0.4 mL) was added followed by 0.2 mL of BSC (benzene sulphonyl chloride). BSC solution was slowly mixed using a vortex mixer and cooled on ice. The developed yellow colour was measured at 410 nm using UV-visible spectrophotometer (UV-1700 Shimadzu, Japan). The percentage (%) of ACE inhibition was calculated with the following equation

$$\text{Percentage (\%)} \text{ inhibition of ACE} = [(A_1 - A_s) / (A_1 - A_2)] \times 100$$

Where, A_1 : Absorbance of the ACE solution without inhibitor

A_s : Absorbance of the ACE solution with inhibitor (MEAL or captopril)

A_2 : Absorbance of HHL solution (a buffer was added instead of the ACE solution and sample)

IC_{50} values ($\mu\text{g/mL}$) for ACE were determined by the same way as described in the above assays.

4.7. Kinetics of ACE inhibition

Here the mode of inhibition mechanism of ACE by MEAL was investigated using enzyme kinetics theory and Lineweaver Burk's method was used to evaluate kinetic parameters [41]. Kinetic studies provide

information on substrate and product affinity to the enzyme. Knowledge of the dynamic properties of enzyme catalysis is a prerequisite for the design of inhibitors against ACE. In kinetic study, the same reaction condition as mentioned in ACE inhibitory activity assay, was applied and the enzyme activity was measured at various substrate (HHL) concentrations (2, 4 and 6 mM) in the presence of the inhibitor at three different concentrations (0, 100 and 200 µg/mL). The rate of formation of HA (hippuric acid) was determined in the presence of different concentrations of MEAL and overall data were used to construct the Lineweaver-Burk plot from which mode of inhibition was determined.

4.8. GC-MS analysis of MBLE

The bioactive compounds of MEAL extract were investigated through GC-MS (Gas chromatography-mass spectrometry) with electron impact ionization (EI) method on gas chromatography (GC-17A, Shimadzu Corporation, Kyoto, Japan), coupled with a mass spectrometer (GC-MSTQ 8040, Shimadzu Corporation, Kyoto, Japan). This instrument was equipped with a Rxi-5MS fused silica capillary column (5% diphenyl/95% dimethyl polysiloxane) and AOC- 20i+s (autosampler) of 0.25 mm diameter, 30 m length, and 0.25 µm film thickness. The injector temperature was maintained at 270°C and 2 µL of the samples was injected neat, with a split ratio of 1: 10. Here helium was used as carrier gas at flow rate of 1.0 mL min⁻¹. Spectra were scanned from 20 to 550 *m/z* at 2 scans s⁻¹. The constituents were identified after comparison with those available in the computer library (NIST 08-S) attached to the GC-MS instrument.

2.9. Statistical analysis

All values were expressed as mean ± SD (Standard Deviation). Statistical analysis was performed with one way analysis of variance (ANOVA) using SPSS statistical software of 16 version. The means were separated by Independent-sample T-test and P<0.05 were considered to be statistically significant.

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