INHIBITORY ACTIVITIES OF OCIMUM SANCTUM AND ITS FLAVONOID CONSTITUENTS ON ANGIOTENSIN-CONVERTING ENZYME

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Abstract:
As part of our ongoing isolation of antihypertensive agents derived from natural sources, the bioactivity of the ethanolic extracts was screened for their inhibitory activities against angiotensin converting enzyme (ACE) inhibitory assays. Ocimum sanctum exhibited promising inhibitory activity against ACE and Total ROS. Among the different solvent-soluble fractions obtained from the ethanolic extract, the dichloromethane (CH2Cl2) fraction was found to cause the most potent scavenging, or inhibitory activities, against total ROS with the respective IC50 values of 3.21 ± 0.03 μg/mL. Likewise, the dichloromethane fraction also exhibited potent inhibitory activities against ACE with IC50 values of 16.19 ± 0.12 μg/mL. Silica gel column chromatography of the dichloromethane fraction yielded two flavonoids, Cirsilineol and Isothymusin, based on the comparison with reported 1H- and 13C-NMR spectroscopic data. All of the compounds displayed concentration dependent in vitro inhibitory activity toward the ACE and Total ROS. Among them, Cirsilineol exhibited the potential inhibitory activity toward ACE with the respective IC50 values of 12.64 ± 0.02 μM. Further inhibitory kinetics analyzed from Lineweaver-Burk plots showed Cirsilineol to be a competitive inhibitor with a Kᵢ value of 8.2 × 10⁻⁵ M. In conclusion, we identified significant ACE inhibitors from Ocimum sanctum that could have value as new multi-targeted compounds for antihypertensive agents.

Keywords: O. sanctum; Hypertension; Antioxidant; Angiotensin Converting Enzyme (ACE); Total Reactive Oxygen Species (ROS).

Please cite this article in press as Howlader Saurav et al, Inhibitory Activities of Ocimum Sanctum and Its Flavonoid Constituents on Angiotensin-Converting Enzyme, Indo Am. J. P. Sci, 2017; 4(10).
1. INTRODUCTION:
Angiotensin-converting enzyme (ACE, peptidylpeptide hydrolase EC3.4.15.1) is a zinc-containing metalloenzyme that performs important physiological functions in the pathogenesis of cardiovascular and renal diseases, as well as in the regulation of blood pressure. In particular, a variety of cardiometabolic syndromes are known to be operant in hypertension. ACE catalyzes the conversion of rennin-induced decapeptide, angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), into the active vasoconstrictor octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). ACE has also been shown to catalyze the degradation of bradykinin, a vasodilator in the kallikrein-kinin system. Furthermore, ACE has been implicated in cell oxidative stress, augmenting the generation of reactive oxygen species (ROS) and peroxynitrite, and also in thrombosis during which it induces platelet activation, aggregation, and adhesion [1]. ACE inhibitors may, to some degree, prevent hypertension and other cardiovascular and renal diseases, as well as oxidative stress-associated diseases. It has been reported, however, that synthetic ACE inhibitors can have certain undesirable side effects like coughing, taste disturbances, hyperpotassemia, and skin rashes [2]. Due to these side effects, as well as considering the preventive potentials of bioactive sources discovered thus far, such as in foods, herbs, and seaweeds, a great deal of interest has begun to focus on the possibility of deriving ACE inhibitors from natural products [3,4]. Several classes of ACE inhibitory compounds have been discovered, including the alkaloids [5], tannins [6,7], flavonoids [8-10], xanthones [11], terpenoids [3], peptides [4], and caffeoylquinic acid derivatives [6].

A plenty of traditional herbal medicinal practices have been adopted for the diagnosis, prevention and treatment of Hypertension. The Ocimum sanctum belonging to family Lamiaceae is medicinally used in diabetes, digestive, diuretic, cardiopathy, haemopathy, leucoderma, asthma, bronchitis, catarhall fever, otalgia, hepatopathy, lumbago, ophthalmia, gastropathy in children, GIT disorders, ringworm, verminosis and skin disease [12-17]. O. sanctum also showed antioxidant, lipid-lowering [18], anti-metastatic [19], antifungal [20], antibacterial [21], antimicrobial [22], wound healing [23] and neuroprotective [24] activities. However, there have been no studies on O. sanctum that show which active components are responsible for ACE as well as Total ROS inhibitory activities. The objective of the present study was to investigate the antihypertensive activity of the different fractions of the ethanolic extract of the leaves of O. sanctum using in vitro models. Our data suggest that O. sanctum may perhaps represent a new way for the prevention and treatment of hypertension through oxidative damage-associated diseases.

2. MATERIALS AND METHODS:
2.1. General Experimental Procedures
Column chromatography was conducted using silica (Si) gel 60 (70–230 mesh, Merck, Darmstadt, Germany), Si gel 60 (230–400 mesh, Merck, Darmstadt, Germany), Sephadex LH20 (20–100 µm, Sigma, St. Louis, MO, USA), Lichroprep RP-18 (40–63 µm, Merck, Darmstadt, Germany). All thin layer chromatography (TLC) was conducted on pre-coated Merck Kiesel gel 60 F254S plates (20 × 20 cm, 0.25 mm, Merck) and RP-18 F254S plates (5 × 10 cm, Merck), using 10% H2SO4 as the spray reagent.

2.2. Chemicals and Reagents
ACE (1 Unit, rabbit lung), FAPGG (N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly), DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate), captopril and trolox were purchased from Sigma Chemical Company (St Louis, MO, USA). All chemicals and solvents used in the assays were of reagent grade, and were purchased from commercial sources.

2.3. Plant Material
The plant sample of O. sanctum leaves were collected in July, 2016 from local area of Bangladesh. The plant was identified by Bangladesh National Herbarium, Dhaka, where a voucher specimen (20160720) has been deposited. At first, Leaves were washed properly to remove dirty materials and air-dried for several days. These were then ground with a hammer grinder for better grinding. The dried leaves were ground into a coarse powder. Then, the dried powder was preserved in an airtight container.

2.4. Extraction, Fractionation and Isolation of O. sanctum
Dried powder of O. sanctum was refluxed with 70% EtOH (3 × 3 L) for 3 h, and each filtrate was concentrated until dry in vacuo at 40˚C, resulting in EtOH extract (250.0 g). This extract was suspended in distilled H2O and then successively partitioned with CH2Cl2, EtOAc, and n-BuOH, to yield the CH2Cl2 (60.6 g), EtOAc (49.5 g), and n-BuOH (30.6 g) fractions, respectively, as well as an H2O residue (92.0 g). The active CH2Cl2 fraction (60.6 g) obtained from O. sanctum was subjected to chromatography on a silica gel column, with CH2Cl2-MeOH (100:1 to 5:1) as the eluent, yielding eighteen subfractions (OS01-OS18). Repeated column chromatography of OS04 (10.30 g) was conducted with a solvent mixture of CH2Cl2 and MeOH, yielding ten subfractions (OS0401-OS0410). OS0402 (0.23 g) was purified on an RP-18 column and eluted with aqueous MeOH (20% MeOH-100% MeOH, gradient elution) to yield flavonoid compound, Cirisinole. Subfraction OS05 was similarly chromatographed and conducted with a solvent mixture of CH2Cl2 and MeOH, yielding
eight subfractions (OS0501(OS0508). OS0503 (0.35 g) was purified on an RP-18 column and eluted with aqueous MeOH (10% MeOH-100 % MeOH, gradient elution) to yield flavone compound, Isothymusin. The chemical structure of this compound was identified by spectroscopic methods, including H- and C-NMR. The structure is shown in Fig. 1.

Compound 1, (Cirsilineol): 1H NMR (500 MHz, acetone-d6): δ 12.96 (s, 1 H, 5-OH), 7.63 (m, 2 H, H-2', 6'), 7.08 (d, J = 8.2 Hz, 1 H, H-5'), 6.84 (s, 1 H, H-8), 6.74 (s, 1 H, H-3), 3.99 (s, 3 H, 3'-OMe), 3.97 (s, 3 H, 7-OMe), 3.80 (s, 3 H, 6-OMe); 13C NMR (125 MHz, acetone-d6): δ 183.60 (C-10), 165.27 (C-2), 144.6 (C-7), 136.1 (C-4), 130.6 (C-3'), 116.47 (C-6'), 110.59 (C-2'), 106.57 (C-10), 104.26 (C-3), 92.00 (C-8), 60.60 (6-OMe), 56.87 (7-OMe), 56.68 (3'-OMe).

Compound 2, (Isothymin): 1H NMR (400 MHz, DMSO-d6): δ 3.80 (3H, s, 6-OMe), 3.92 (3H, s, 7-OMe), 6.82 (1H, s, H-3), 6.92 (2x1H, d, J 8.7 Hz, H-2', H-6'), 8.01 (2x1H, d, J 8.7 Hz, H-2', H-6'), 12.43 (1H, s, 5-OH); 13C-NMR (100 MHz, DMSO-d6): δ 60.4 (6-OMe) 61.1 (8-OMe), 102.4 (C-3), 106.3 (C-10), 115.9 (C-3', C-5'), 121.2 (C-1'), 128.7 (C-2', C-6'), 130.6 (C-8), 136.1 (C-6), 141.3 (C-9), 144.6 (C-5), 148.0 (C-7), 161.3 (C-4'), 164.2 (C-2), 182.7 (C-4).

2.5. ACE inhibitory activity assay

The ACE inhibitory activity assay was conducted according to Hou et al. [25] modified to use FAPGG as the substrate. In brief, FAPGG (0.5 mM) and various concentrations of the samples were completely dissolved in 50 mM Tris-HCl buffer (pH 7.5). Twenty microliters of ACE (1 U/mL dissolved in 50 mM Tris-HCl buffer) was then mixed with 200 µL of various concentrations of the samples as experimental samples, or with 50 mM Tris-HCl buffer as a negative control. After addition of 1 mL of 0.5 mM FAPGG to the reaction mixture, optical density was determined at a wavelength of 345 nm, at time intervals of 0, 5, 30, and 60 min. The inhibitory activity of the crude extracts was represented as a percentage inhibition at 163.93 µg/mL concentration. The ACE inhibitory activities were expressed as 50% inhibition concentration (IC50) of fractions (µg/mL), and compounds (µM). The percentage inhibition was calculated. In order to prevent interference from the inherent color of the samples, all optical density (OD) values at 0 min (OD0min) were subtracted from the values at 60 min (OD60min). The values of percentage inhibition were then calculated using the equation ([1 - (ODsample/ODcontrol) × 100]. An antihypertensive agent, captopril was used as a positive control. All experiments were conducted three times.

2.6. Assay for inhibition of total ROS generation

The generation of ROS was assessed using the ROS-sensitive fluorescence indicator DCFH-DA [26]. Male Wistar rats weighing 150-200 g were sacrificed by decapitation and the kidneys were quickly removed and rinsed in ice-cold buffer [100 mM Tris, 1 mM EDTA, 0.2 mM PMSF, 1 mM pepstatin, 2 µM leupeptin, 80 mg/L trypsin inhibitor, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate (pH 7.4)]. The tissues were immediately frozen in liquid nitrogen and stored at -80°C. 10 µL of each test sample (f.c.25 µg/mL) was added to 190 µL of kidney postmitochondrial fraction in a 50 mM potassium phosphate buffer. Then, the mixtures were loaded with 50 µL of DCFH-DA (12.5 mM) in a potassium phosphate buffer and shaken for 5 min. Finally, the fluorescence of 2′,7′-dichlorodihydrofluorescein (DCF), the oxidation product of DCFH-DA was measured on a microplate fluorescence spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) for 30 min at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Trolox was used as the positive control.

2.7. Kinetic Analysis

The reaction mixture consisted of four different concentrations of FAPGG (0.1 to 1 mM) as a
substrate, and ACE in 50 mM Tris-HCl buffer. Each sample of several concentrations was added to the re-action mixture, respectively. The Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) of ACE were deter-mined by Lineweaver-Burk plots. The velocity equation for the competitive inhibition in reciprocal form was: $1/V = K_m/V_{max} (1 + [I]/K_i) 1/[S] + 1/V_{max}$. The inhibition constants ($K_i$) of the competitive inhibitors were calculated by the following equation: $K_{mapp} = K_m[1 + ([I]/K_i)]$, where $K_{mapp}$ is the apparent $K_m$ in the presence of any inhibitor concentration.

2.8. Statistical Analysis
One-way ANOVA and Student’s t test were used to determine the statistical significance of differences between values for various experimental and control groups (Systat; Evaston, IL, USA). Each result is expressed as the mean ± SEM of triplicates.

3. RESULTS
3.1. ACE inhibitory activities of the EtOH extract and its solvent soluble fractions from O. sanctum
The inhibitory activities of the EtOH extract against ACE are shown in Table 1. The EtOH extract showed potential inhibitory activity against ACE with IC$_{50}$ values of 64.85 ± 0.56 μg/mL compared to the positive control Captopril with IC$_{50}$ values of 2.30 ± 0.02 (ng/mL). The EtOH extract of O. sanctum was dissolved in H$_2$O and successively partitioned with CH$_2$Cl$_2$, EtOAc, and n-BuOH to obtain different solvent-soluble fractions, which were individually tested for ACE inhibitory activity. As shown in Table 1, the CH$_2$Cl$_2$ and EtOAc fractions showed the highest ACE inhibitory activity with IC$_{50}$ values of 16.91 ± 0.12 and 47.57 ± 0.21 μg/mL, respectively.

<table>
<thead>
<tr>
<th>Extract/Fractions</th>
<th>IC$_{50}$ values (μg/mL)$^a$ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH extract</td>
<td>64.85 ± 0.56</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$ fraction</td>
<td>16.91 ± 0.12</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>47.57 ± 0.21</td>
</tr>
<tr>
<td>n-BuOH fraction</td>
<td>92.76 ± 2.23</td>
</tr>
<tr>
<td>H$_2$O fraction</td>
<td>NA</td>
</tr>
<tr>
<td>Captopril$^b$</td>
<td>2.30 ± 0.02</td>
</tr>
</tbody>
</table>

$^a$ The concentration that caused 50% inhibition (IC$_{50}$) is given as the mean ± SEM of triplicate experiments

Table 2: Total ROS scavenging activities of the ethanolic extract and its solvents-soluble fractions from O. sanctum

<table>
<thead>
<tr>
<th>Extract/Fractions</th>
<th>IC$_{50}$ values (μg/mL)$^a$ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH extract</td>
<td>18.81 ± 0.18</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$ fraction</td>
<td>3.21 ± 0.03</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>17.56 ± 0.23</td>
</tr>
<tr>
<td>n-BuOH fraction</td>
<td>42.15 ± 1.21</td>
</tr>
<tr>
<td>H$_2$O fraction</td>
<td>NA</td>
</tr>
<tr>
<td>Trolox$^b$</td>
<td>6.23 ± 0.06</td>
</tr>
</tbody>
</table>

$^a$ The concentration that caused 50% inhibition (IC$_{50}$) is given as the mean ± SEM of triplicate experiments

$^b$ Used as positive control in Total ROS scavenging assay
3.2. Total ROS scavenging activity of the EtOH extract as well as different fractions from O. sanctum

Total ROS scavenging activities of the EtOH extract and its different solvent-soluble fractions of O. sanctum are presented in Table 2. CH$_2$Cl$_2$ fraction displaying the highest inhibitory activity with an IC$_{50}$ value of 3.21 ± 0.03 µg/mL compared to the positive control Trolox with an IC$_{50}$ value of 6.23 ± 0.06 µg/mL. In addition, the EtOAc and n-BuOH fractions also showed significance inhibitory activity with corresponding IC$_{50}$ values of 17.56 ± 0.23 and 42.15 ± 1.21 µg/mL, respectively.

3.3. Antioxidant and Angiotensin Converting Enzyme (ACE) inhibitory activities of compound isolated from O. sanctum

The inhibitory activities of the isolated compounds against ACE and Total ROS are expressed as IC$_{50}$ values in Table 3. Among the tested compounds, Cirsilineol showed the most potent inhibitory activity against ACE with an IC$_{50}$ value of 12.64 ± 0.02 µM, whereas captopril had an IC$_{50}$ value of 25.26 ± 0.01 nM. Isothymusin also showed significant ACE inhibitory activity, with IC$_{50}$ values of 34.25 ± 0.15 µM, respectively. In addition, both compounds exhibited significant inhibitory effects against Total ROS, with scavenging potencies as indicated by IC$_{50}$ values of 5.40 ± 0.10, and 9.41 ± 0.32 µM, respectively whereas IC$_{50}$ values of the positive controls, trolox was 4.25 ± 0.39 µM.

3.4. Enzyme kinetic analysis with ACE

The inhibition kinetics for compound, Cirsilineol was analyzed by Lineweaver-Burk plots, as shown in Fig. 2. The lines, which were obtained from the uninhibited enzyme and from the different concentrations of cirsilineol, intersected to the left of the 1/V-axis above the 1/S-axis. The results indicate that cirsilineol exhibited a competitive-type of inhibition with respect to the substrate (FAPGG). The equilibrium constant for inhibitor binding ($K_i$) of cirsilineol was estimated to be 8.2 × 10$^{-5}$ M. This competitive inhibition implies that cirsilineol inhibit ACE activity by competing with the substrate for active sites.

### Table 3: Antioxidant and Angiotensin-converting enzyme inhibitory activities of compound isolated from O. sanctum

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (µM) ± SEM$^a$</th>
<th>Total ROS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACE</td>
<td></td>
</tr>
<tr>
<td>Cirsilineol</td>
<td>12.64 ± 0.02</td>
<td>5.40 ± 0.10</td>
</tr>
<tr>
<td>Isothymusin</td>
<td>34.25 ± 0.15</td>
<td>9.41 ± 0.32</td>
</tr>
<tr>
<td>Captopril$^b$</td>
<td>25.26 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Trolox$^c$</td>
<td>4.25 ± 0.39</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The concentration that caused 50% inhibition (IC$_{50}$) is given as the mean ± SEM of triplicate experiments

$^b$ Used as positive control in ACE inhibitory assay

$^c$ Used as positive control in Total ROS inhibitory assay

### Table 3: Kinetic studies of Cirsilineol for ACE

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_i$$^a$</th>
<th>Inhibition type$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirsilineol</td>
<td>8.2 × 10$^{-5}$</td>
<td>Competitive</td>
</tr>
</tbody>
</table>

$^a$ Inhibition constants ($K_i$)

$^b$ Inhibition type were determined by interpretation of the Lineweaver-Burk plot

Fig. 2: Lineweaver-Burk plots for the inhibition of ACE by Cirsilineol.
DISCUSSION:
Hypertension is considered to be one of the strongest cardiovascular risk indicators, and is an increasingly serious public health problem around the world (WHO, 2013). The regulation and control of blood pressure are exerted by a number of cellular and humoral systems. One of the important parameters determining blood pressure is the peripheral vascular resistance which is effected by many regulators such as angiotensin II, endothelin-I, vasopressin, bradykinin, endothelin-derived nitric oxide and prostacyclin [27]. Among these, angiotensin II is a potent vasoconstrictor and is responsible for the elevation of blood pressure. This peptide is activated by the angiotensin I converting enzyme (ACE). ACE is a zinc-dipeptidyl dipeptidase, and activates angiotensin II by the removal of the carboxy-terminal dipeptide of angiotensin I. Angiotensin II also stimulates the synthesis and the release of aldosterone from the adrenal cortex, and this event increases blood pressure via sodium retention [28]. Therefore, the inhibition of ACE has been considered as one of the effective ways for the treatment of hypertension [29]. In addition, ACE inhibitors have been emphasized as potential therapeutic candidates in the chronic treatment of various cardiovascular diseases [30]. A number of screening studies on ACE inhibitory activities derived from plants have been reported [31, 32] and several classes of plant-derived secondary metabolites have been described as ACE inhibitors [31]. It is also noteworthy that a tetrameric procyanidin glycoside, which is closely related to in vitro ACE-inhibiting procyanidins [28], has shown in vivo the same ACE-inhibitory activity as the clinically used captopril [33].

_Ocimum sanctum_ L. (Lamiaceae), a well-known herbal medicine, is widely distributed throughout the world [34]. Its leaves have long been used to treat a variety of ailments, including ozena, skin diseases, and gastric and hepatic disorders and are used as a diaphoretic, an antiperiodic, and an expectorant. Several types of constituents have been isolated from _O. sanctum_ including terpenoids, phenolic derivatives, hydroxycinnamic acid derivatives, benzoic acid derivatives, flavonoids and their glycosides, and eugenol and eugenol glycosides. Some of these compounds have been reported to exhibit antioxidant, antimicrobial, anti-inflammatory, antistress, anthelmintic, and radio-protective activities [35-38]. In our present study, we found that ethanol extract of _O. sanctum_ showed potent inhibitory activities against angiotensin converting enzyme (ACE) and antioxidant scavenging activity. Among the tested fractions, CH₂Cl₂ fraction showed potential activity compared to other polar fractions. The CH₂Cl₂ fraction was found as the most active fraction by ACE inhibitory assays. The CH₂Cl₂ fraction was also shown to possess strong inhibitory activities against total ROS scavenging assay. Considering the inhibitory potential, CH₂Cl₂ fraction was selected for chromatographic separation in order to determine the active compounds from _O. sanctum_. Repeated chromatography of the CH₂Cl₂ fraction yielded two flavonoid compounds, Cirsilineol and isothymusin, was found to be the most active compound in the ACE inhibitory assays as well as total ROS inhibitory assay. ACE is a zinc-containing peptidyl dipeptidase hydrolase [39]. The active site of ACE is known to consist of three parts; a carboxylate binding functionality such as the guanidinium group of Arg, a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues, and zinc ion. The zinc ion coordinates to the carbonyl group of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucelophilic attack. Therefore, some flavonoids [40] were suggested to show in vitro activity via the generation of chelate complexes within the active center of ACE. Free hydroxyl groups of phenolic compounds are also suggested to be important structural moieties to chelate the zinc ions, thus inactivating the ACE activity [41]. Since cirsilineol and isothymusin contains aromatic hydroxyl groups, this hydroxyl groups may show an ACE inhibitory activity due to the generation of chelate complexes with zinc ions within the active center of ACE. Therefore, our results clearly demonstrated that _O. sanctum_ and its constituent have great value in the development of therapeutic and preventing agents for hypertension. Further in vivo and cell-based studies are needed to clarify the detailed mechanism of action of these compounds.

CONCLUSION:
The present bioactivity-guided fractionation and isolation study of _O. sanctum_ on ACE inhibition demonstrated that the isolated constituents exerted potential ACE inhibitory effects, and total ROS scavenging effects. In particular, the ACE along with total ROS inhibitory activities of the isolated compounds was investigated for the first time. The findings of the present study demonstrated that _O. sanctum_ and its isolated constituents might act as a therapeutic or preventive agent for hypertension by alleviating oxidative stress.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

ACKNOWLEDGEMENT
The authors are highly thankful to Department of Pharmacy, Daffodil International University for their consent help and support.
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