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Ocimum sanctum L. a potential angiotensin converting enzyme (ACE) inhibitor useful in hypertension

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The main objective of this study was to determine the *in vitro* Angiotensin Converting Enzyme (ACE) inhibition and antioxidant activity of standardized extract of the *Ocimum sanctum* L. leaves and its fractions. The ACE inhibition activity and antioxidant activity were investigated by UV method. In ACE inhibition method, the synthetic substrate hippuryl-L-histidyl-L-leucine was allowed to react with a test sample containing ACE, to produce hippuric acid. Dried hippuric acid was dissolved in distilled water, the absorbance of the solution was determined in the ultraviolet region and from the concentration of hippuric acid and the ACE inhibition activity was calculated. The antioxidant activity of the plant extract/fractions were examined on the basis of the scavenging effect on the stable DPPH free radical activity. Different concentrations of methanol extract of *O. sanctum* L. and eugenol were subjected to HPLC analysis using the mobile phase (methanol: water: acetic acid; 60 %: 40 %: 1 %). *O. sanctum* L. oil, eugenol and ethyl acetate fractions showed the maximum ACE inhibition activity in a concentration-dependent manner with IC_{50} value of 32.11 ± 3.6, 42.16 ± 2.7 and 56.83 ± 2.8 µg/mL, respectively. Strong DPPH radical scavenging was also found in *O. sanctum* L. oil, methanol extract and ethyl acetate fractions, showing IC_{50} value of 40.31± 3.5µg/mL, 105.62 ± 4.6 and 145.31 ± 5.8µg/mL, respectively. *O. sanctum* L. extract/fractions, eugenol and *O. sanctum* L. oil inhibited ACE in concentration-dependent manner.

Keywords: ACE inhibition, *Ocimum sanctum* L., Eugenol, Antioxidant, HPLC.

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Introduction

Hypertension is the most common serious persistent health problem, which affects 15-20 % of all adults and carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end stage renal disease[1]. In the treatment of hypertension, inhibition of the Angiotensin Converting Enzyme (ACE) is established as one of the modern therapeutic approaches[2]. The renin–angiotensin system plays a vital role in the treatment of hypertension and congestive heart failure[3]. Renin produced from the juxtaglomerular apparatus of the kidney splits angiotensinogen to produce the inactive decapeptide angiotensin I. The latter is then converted to the powerful octapeptide vasoconstrictor, angiotensin II by the action of angiotensin converting enzyme (ACE). Angiotensin II produced both peripheral and central effects[4] and this stimulates secretion in adrenal/cardiovascular tissue, promotes sodium and water retention and causes an increase in renin generation in kidneys. ACE also catalyzes the degradation of bradykinin, a vasodialator. ACE is implicated in cell oxidative stress, augmenting the generation of reactive oxygen species, peroxynitrile, and also in thrombosis, during which ACE induces platelets activation, aggregation and adhesion.

Oxidative stress, which results in an excessive generation of reactive oxygen s (ROS), plays a key role in the pathogenesis of hypertension. The modulation of the vasomotor system involves ROS as mediators of vasoconstriction induced by angiotensin II. Treatment with antioxidants may lower blood pressure and improve vascular structure and function in hypertension condition. Association between antioxidants and decrease in blood pressure has been reported**.** Use of antioxidant has gained considerable interest as protecting agents against vascular endothelial damage. Antioxidants can counteract ROS effects and its vascular damage and may be a possible therapeutic strategy to prevent or treat ROS-associated cardiovascular disorders[5]. Thus inhibition of ACE plays important role to prevent hypertension, cardiovascular, renal diseases and oxidative stress-associated disease[6].

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Ocimum sanctum L. (Lamiaceae) is considered a sacred plant in the Hindu culture and known as ‘‘Tulsi” or ‘‘Tulasi” in Hindi or Holy Basil in English. Leaves of Tulsi are very rich in oils, eugenol, euginal (phenolic compounds), ursolic acid (pentacyclic triterpene acid), carvacrol (monoterpenolphenol), limelool (terpene alcohol) and sterols[7]. The important ingredients present in the leaves extract are triterpenes, flavonoids and eugenol. It has also been suggested to possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, analgesic, adaptogenic and diaphoretic actions[8, 9]. The present investigation has been carried out to evaluate the role of *O. sanctum* L. leaves extract/fractions/oil in Angiotensin converting enzyme inhibition.

Materials and Methods

Plant materials and chemicals

Leaves of *O. sanctum* L. were purchased from an authenticated vendor and identified. Voucher specimen (SNPS/JU/2010/1055) has been deposited at the School of Natural Product Studies, Jadavpur University, Kolkata, India.

ACE (from rabbit lung; 3.4 units/mg of protein), hippuryl-L-histidyl-L-leucine (HHL), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and eugenol, were purchased from Sigma Chemical Co (St. Louis, MO, USA). Methanol and all other analytical grade organic solvents (HPLC grade) were procured from Merck, India.

Extraction and fractionation

Shaded dried leaves (100 g) were grinded to a coarse powder and extracted with methanol for 48 h at room temperature. The resultant material was filtered and evaporated under reduced pressure using rotary evaporator. The step executed three times to afford the crude methanolic extract (yield 13.8 % w/w). This *O. sanctum* L. methanol extract (OSME; 4 % w/w) was suspended in water and fractionated successively with ethyl acetate and n-butanol. All the fractions were evaporated under reduced pressure by using rotary evaporator (EYELA, Tokyo, Japan) to afford ethyl acetate fraction (OSEF; 1.7 % w/w), n-butanol fraction (OSBF; 1.3 % w/w) and aqueous fraction (OSAF; 0.6 % w/w). The extract and all the fractions were stored at 4 ºC until analysis.

Isolation of oil

The fresh leaves (100 g) of *O. sanctum* L. was hydro distilled in a Clevenger’s apparatus for 6 h and yellow colored oil with characteristic odor and sharp taste was obtained [10]. The crude oils was dried over anhydrous sodium sulphate to remove traces of moisture and stored in a sterilized vial in a refrigerator in the dark at 4 °C until use. The chemical analysis of *O. sanctum* L.oil was made with GC-MS.

Determination of ACE inhibition activity

ACE inhibitory activity was assayed according to the method described by Cheung and Cushman[11] and Chaudhary *et al*[12] with some modifications. This method was based on the liberation of hippuric acid from HHL catalyzed by the ACE. In brief, 50 µL of ACE (25 mU/mL) with 50 µL of the sample solution (at varied concentrations of 20-100 µg/mL) was pre-incubated at 37ºC for 10 min. Afterward 150 µL of the substrate solution (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) was added to the above mixture and incubated for 30 min at 37 ºC. The reaction was terminated by addition of 250 µL of 1.0 M HCl. To the resulting solution 0.5 mL of ethyl acetate was added and centrifuged (800 g) for 15 min. 0.2 mL of the upper layer was collected into a test tube and evaporated at room temperature for 2 h under vacuum. The hippuric acid was dissolved in 1.0 mL of distilled water and absorbance was measured at 228 nm using an UV-spectrophotometer (Cecil, CE 7200 England). All the experiments were performed in triplicate and in two independent experiments. Captopril was used as a standard at concentration of 3.6 ng/mL[13]. The percentage of inhibition (ACEI) was calculated using the formula:

$$\text{Percentage inhibition} = (A-B)/(A-C) \times 100$$

Where, A is the optical density at 228 nm with ACE but without Inhibitor.

B is the optical density in the presence of both ACE and inhibitor.

C is the optical density without ACE and inhibitor.

Antioxidant activity

The antioxidant activity of the plant extract/fractions/oil was examined on the basis of the scavenging effect on the stable DPPH free radical activity[14]. DPPH (0.05 mM) (300 µL) was added to 40 µL of extract solution with different concentrations (10-500 µg/mL). DPPH solution was freshly prepared and kept in the dark at 4°C. Methanol 96 % (2.7 mL) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min.

and absorbance was measured by using an UV-spectrophotometer (Cecil, CE 7200 England) at 517 nm. Methanol was used to set the absorbance zero. A blank sample containing the same amount of methanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples were expressed as percentage of inhibition and calculated according to the following equation.

Percent (%) inhibition of DPPH activity = [(AB − AA) / AB] x 100

Where, AA and AB are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50 % inhibition was determined and represented as IC_{50} value for each of the test solutions. Ascorbic acid was used as standard at concentration of IC_{50} = 19.36 ± 1.75 µg/mL.

HPLC analysis

The methanol extract was standardized for the presence of eugenol. The OSME was precisely measured, extracted with methanol in a sonicator and filtered through 0.45 µm filter. An aliquot of 20 µL was injected in to the HPLC column (Waters Spherisorb C_{18} column, 250 × 4.6 mm, 5 µ) and elution was carried out using methanol: water: acetic acid (60: 40: 1 v/v) as mobile phase at a flow rate of 1 mL/min., and elute was monitored at 254 nm. The external calibration curve of standard eugenol was prepared in the concentration range of 0.5-15 µg/mL.

Identification of eugenol in volatile oil of *O. sanctum* L. by GC-MS analysis

Isolated oil of *O. sanctum* L. was analyzed by GC-MS (GC-MS QP5050, Shimadzu, Kyoto, Japan). A capillary column DB5 (30 m × 0.32 mm i.d., 0.25 µm film thickness; J&W Scientific Inc., Folsom, CA, U.S.A.) with cross-linked 5 % phenyl methyl silicone was used. Helium gas was used as a mobile phase at a flow rate of 2.2 mL/min. The oven temperature was isothermal at 40°C for 5 min, and then gradually increased to 220°C at a rate of 5°C per min and kept isothermal for 10 min. Injection and interface temperatures were 220°C and 200°C, respectively. Injection volume used was 0.5 µL with split ratio of 1:25. The volatile oil diluted in methanol at a concentration of 0.1 mg/mL. The compounds were identified by matching the mass spectra data with that of the standard components[15].

Statistical analysis

The IC_{50} values were expressed as mean ± standard error mean (SEM) by plotting the curve with percentage of inhibition versus concentrations of the individual experiments measured (n = 3). Statistical analysis was performed by one way ANOVA followed by Bonferroni post test using Graph Pad prism version 5.0. As compared to reference standard $P< 0.05$ was considered as significance difference.

Results and Discussion

The present investigation was aimed to evaluate the angiotensin converting enzyme inhibition potential and to standardize the *O. sanctum* L. extract and with the bioactive marker, eugenol. Methanol extract was subjected to standardization by HPLC method and the oil of *O. sanctum* L. was analyzed by GC-MS method. The HPLC quantitative analysis of the eugenol in *O. sanctum* L. extracts showed a linear relationship between peak area and concentration (0.5-15µg/mL) with a correlation coefficient, r^2 = 0.9962. The concentration of eugenol in the extracts was calculated from the experimental peak areas by interpolation to standard calibration curves and % of eugenol was found to be 5.3 % w/w in methanol extract. Retention time of eugenol was found to be 10.06 min (Fig. 1a and 1b). Simultaneously the GC-MS analysis (Fig. 2a & 2b) revealed that the major compound of the oil is eugenol which was identified by comparing mass spectral data with the NIST

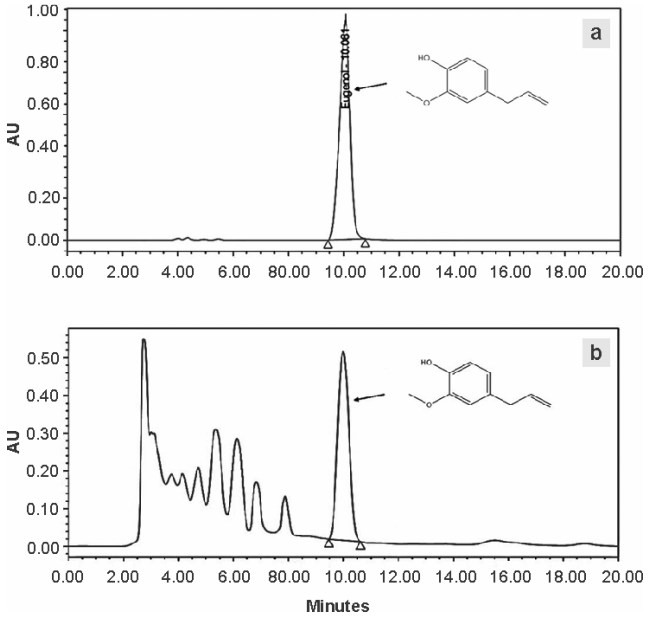

Fig. 1—a. HPLC chromatogram of standard eugenol, b. HPLC chromatogram of methanol extract of *O. sanctum* L

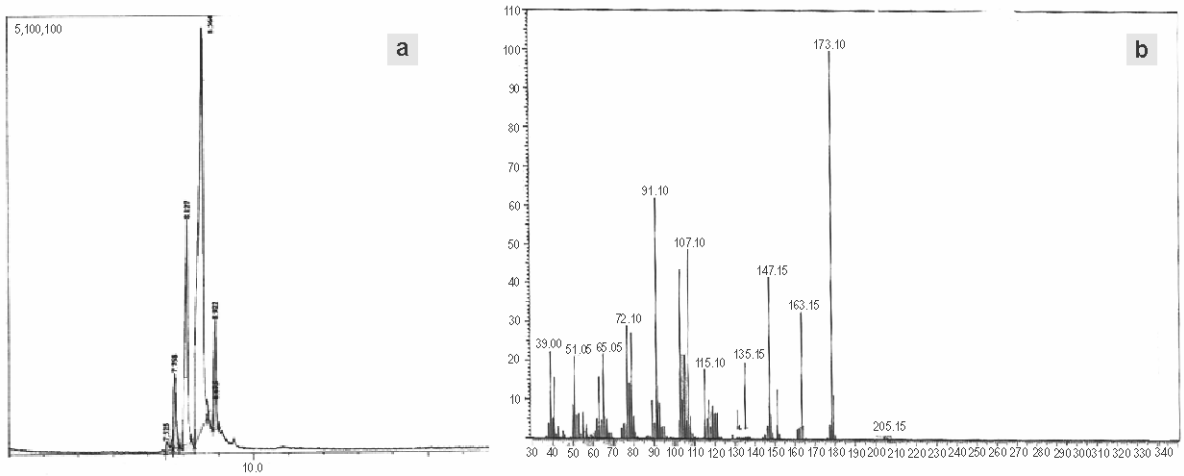

Fig. 2— a. Gas chromatogram and b. -Mass spectra of *O. sanctum* L oil

Table 1 — IC_{50} values of ACE inhibition and antioxidant activity of *O. sanctum* L. extract, fractions, oil and eugenol

Extract/Fractions	ACE inhibition activity (IC_{50}:µg/mL)	Antioxidant activity (IC_{50}: µg/mL)
OSME	79.28 ± 4.9	105.62 ± 4.6
OSEF	56.83 ± 2.8	145.31 ± 5.8
OSBF	69.18 ± 3.2	223.42 ± 7.4
Eugenol	42.16 ± 2.7	186.56 ± 8.3
Oil	32.11 ± 3.6	40.31± 3.5

All values are expressed as mean ± SEM (n=3).

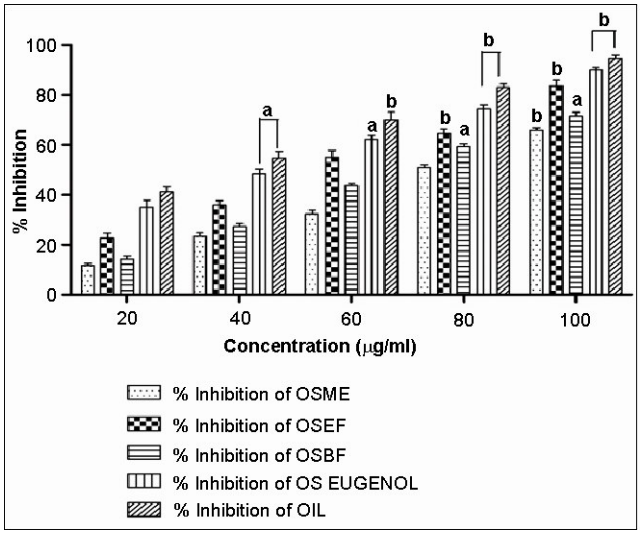

Fig. 3—ACE inhibition activity of *O. sanctum* L. extract, fractions, oil and eugenol. All values are expressed as mean ± SEM (n=3). $^{b}P < 0.01$ and $^{c}P < 0.001$ compared with standard. OSME; methanol extract of *O. sanctum* L., OSEF; ethyl acetate fraction of *O. sanctum* L, OSBF; n-butanol fraction of *O. sanctum* L, OSAF; aqueous fraction of *O. sanctum* L.

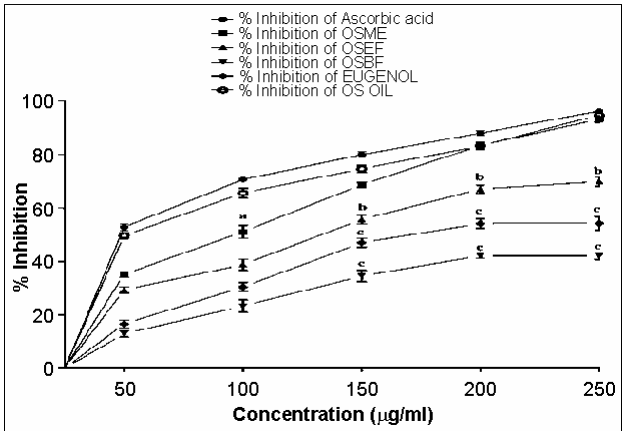

Figure 4—Antioxidant activity of *O. sanctum* L. extract, fractions, oil and eugenol. All values are expressed as mean ± SEM (n=3). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ compared with standard. OSME; methanol extract of *O. sanctum* L., OSEF; ethyl acetate fraction of *O. sanctum* L, OSBF; n-butanol fraction of *O. sanctum* L, OSAF; aqueous fraction of *O. sanctum* L.

library, as well as Kovats indices[16]. The volatile oil showed maximum inhibition and among the extracts, the ethyl acetate fraction was found to be most active (Table 1). The dose response relationships of the ACE inhibition have been shown in Fig. 3. It was found that the activity of the isolated oil showed better activity than extract and other fractions of *O. sanctum* L. leaves. The antioxidant activity of *O. sanctum* L. was established through DPPH. The % inhibition activity of the *O. sanctum* L. oil, and its extract showed significant increment in the enzyme inhibition compared to the fractions (Fig. 4). Interestingly the ACE inhibition potential of the *O. sanctum* L. leaves was found to be directly proportional to the amount of eugenol present in the extract or the oil. This finding signifies that eugenol is abundantly present in

O. sanctum L. leaves and is one of the responsible constituents for the ACE inhibition activity. Isolated oil of *O. sanctum* L. leaves was found to be more active in comparison to the extract/fractions. This may be due the maximum content of eugenol in it.

Conclusion

From this study it can be concluded that, *O. sanctum* L. leaves has potential ACE inhibitory activity and eugenol was present in it as the bioactive constituents. The leaves and its oil may be explored further as a potential lead for development of natural ACE inhibitory agents**.**

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