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EXTRACTION AND IN VITRO SCREENING OF POTENTIAL ACETYLCHOLINESTERASE, BUTYRYLCHOLINESTERASE AND BACE1 INHIBITORS FROM THE LEAVES OF *OCIMUM SANCTUM*

Rajbongshi Lata[1], Rahman Azizur[1], Rahman Mizanur[1], Howlader Saurav[2*]

[1]Department of Pharmacy, Daffodil International University, Dhanmondi, Dhaka

[2]Department of Pharmacy, Southeast University, Dhaka, Bangladesh

Abstract:

As part of our ongoing isolation of cholinesterase (ChE) and β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) inhibitors from natural sources, the bioactivity of the ethanolic extracts was screened for their inhibitory activities against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and BACE1. Ocimum sanctum exhibited promising inhibitory activity against AChE, BChE, BACE1 and peroxynitrite ($ONOO^-$). Among the different solvent-soluble fractions obtained from the ethanolic extract, the dichloromethane (CH_2Cl_2) fraction was found to cause the most potent scavenging, or inhibitory activities, against peroxynitrite ($ONOO^-$) with the respective IC_{50} values of 1.21 ± 0.05 µg/mL. Likewise, the dichloromethane fraction also exhibited potent inhibitory activities against AChE, BChE and BACE1 with IC_{50} values of 2.54 ± 0.03, 13.52 ± 0.13 and 3.05 ± 0.01 µg/mL, respectively. Silica gel column chromatography of the dichloromethane fraction yielded two flavonoids, Cirsilineol and Isothymusin, based on the comparison with reported ^1H- and ^{13}C-NMR spectroscopic data. All of the compounds displayed concentration dependent in vitro inhibitory activity toward the ChEs, BACE1 and peroxynitrite ($ONOO^-$). Among them, Cirsilineol exhibited the potential inhibitory activity toward ChEs with the respective IC_{50} values of 2.95 ± 0.02 and 3.25 ± 0.08 µM, whereas the potential BACE1 inhibitor was Isothymusin with IC_{50} values of 4.45 ± 0.05 µM. In conclusion, we identified significant ChE and BACE1 inhibitors from Ocimum sanctum that could have value as new multi-targeted compounds for anti-AD agents.

Keywords: *O. sanctum; Alzheimer's diseases; Antioxidant; Acetylcholinesterase; Butyrylcholinesterase; β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1).*

Corresponding Author:

Howlader Saurav,

Department of Pharmacy

Southeast University

Dhaka, Bangladesh

E-mail: sauravpharm@gmail.com

QR code

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INTRODUCTION:

Alzheimer's disease (AD) is one of the most common aging-related neurological disorders that usually starts slowly and gets worse over time. AD is correlated with the appearance of neurofibrillary tangles, senile plaques, and loss of neurons in the brain [1]. Although the exact mechanisms of the pathogenesis of this disease remain unclear, several competing hypotheses have been proposed, including cholinergic hypothesis and beta (β)-amyloid cascade hypothesis. According to the cholinergic hypothesis, the memory impairment in AD results from the dysfunctions in the central cholinergic neurotransmission of acetylcholine [2,3]. In particular, cholinesterases (ChEs) serve as the key enzymes which highly implicated in the pathogenesis of AD [4]. The intracellular and extracellular accumulations of Aβ peptides are also believed to play a key role in AD [5,6]. Aβ is formed through the amyloidogenic pathway in which amyloid precursor protein (APP) is sequentially cleaved by β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and γ-secretase [7]. The proteolytic processing of APP increased the production and accumulation of neurotoxic forms of Aβ in the brain.

A plenty of traditional herbal medicinal practices have been adopted for the diagnosis, prevention and treatment of Alzheimer disease. The *Ocimum sanctum* belonging to family Lamiaceae is medicinally used in diabetes, digestive, diuretic, cardiopathy, haemopathy, leucoderma, asthma, bronchitis, catarrhal fever, otalgia, hepatopathy, lumbago, ophthalmia, gastropathy in children, GIT disorders, ringworm, verminosis and skin disease [8-13]. *O. sanctum* also showed antioxidant, lipid-lowering [14], anti-metastatic [15], antifungal [16], antibacterial [17], antimicrobial [18], wound healing [19] and neuroprotective [20] activities. However, there have been no studies on *O. sanctum* that show which active components are responsible for AChE, BChE, BACE1 as well as peroxynitrite ($ONOO^-$) inhibitory activities. The objective of the present study was to investigate the anti-alzheimer 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 source of new way for the prevention and treatment of neuronal disorders such as AD and oxidative damage-associated diseases.

MATERIALS AND METHODS:

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 F_{254} plates (20 × 20 cm, 0.25 mm, Merck) and RP-18 F_{254S} plates (5 × 10 cm, Merck), using 10% H_2SO_4 as the spray reagent.

Chemicals and Reagents

Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (EC 3.1.1.8), acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), eserine, L-penicillamine (L-2-amino-3-mercapto-3-methylbutanoic acid), and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma (St. Louis, MO, USA). A BACE1 FRET assay kit (bsecretase) was purchased from PanVera Co. (Madison, WI, USA). High quality dihydrorhodamine 123 (DHR 123) was purchased from Molecular Probes (Eugene, OR, USA), and $ONOO^-$ was purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). All chemicals and solvents used in the assays were of reagent grade, and were purchased from commercial sources.

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.

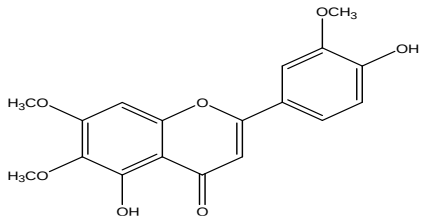
Extraction, Fractionation and Isolation of *O. sanctum*

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 40^0 C, resulting in EtOH extract (250.0 g). This extract was suspended in distilled H_2O and then successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH, to yield the CH_2Cl_2 (60.6 g), EtOAc (49.5 g), and *n*-BuOH (30.6 g) fractions, respectively, as well as an H_2O residue (92.0 g). The active CH_2Cl_2 fraction (60.6 g) obtained from *O. sanctum* was subjected to chromatography on a silica gel column, with CH_2Cl_2-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 CH_2Cl_2 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, Cirsilineol. Subfraction OS05

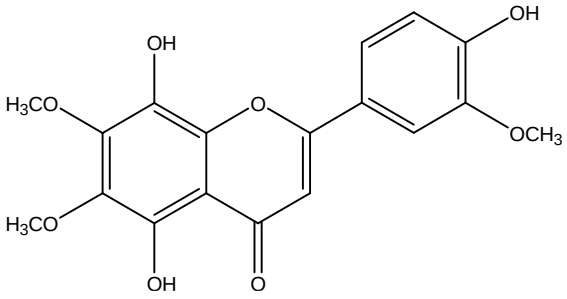
was similarly chromatographed and conducted with a solvent mixture of CH_2Cl_2 and MeOH, yielding eight subfractions (OS0501-OS0508). OS0503 (0.35 g) was purified on an RP-18 column and eluted with aqueous MeOH (10% MeOH-100 % MeOH, gradient elution) to yield flavone compound, Isothymusin. The chemical structure of this compound was identified by spectroscopic methods, including ^{1}H- and ^{13}C-NMR. The structure is shown in Fig. 1.

Compound **1, (Cirsilineol)**: ^{1}H NMR (500 MHz, acetone-*d*6): 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); ^{13}C NMR (125 MHz, acetone-*d*6): δ 183.60 (C-4), 165.27 (C-2), 160.13 (C-7), 154.13 (C-5), 154.01 (C-9), 151.61 (C-4′), 148.98 (C-3′), 133.55 (C-6), 123.60 (C-1′), 121.45 (C-6′), 116.47 (C-5′), 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, (Isothymusin)**: ^{1}HNMR (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 (2×1H, d, J 8.7 Hz, H-3', H-5'), 8.01 (2×1H, d, J 8.7 Hz, H-2', H-6'), 12.43 (1H, s, 5-OH); ^{13}C-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).



Cirsilineol (**1**)



Isothymusin (**2**)

Fig. 1: Structure of Cirsilineol and Isothymusin

In vitro ChEs inhibitory activity assay

The inhibitory activities of ChEs were measured using the spectrophotometric method developed by Ellman et al. [21]. ACh and BCh were used as the substrates to assess the inhibitory activity of AChE and BChE, respectively. The reaction mixture contained: 140 mL of sodium phosphate buffer (pH 8.0), 20 mL of test sample solution and 20 mL of either AChE or BChE solution, which were mixed and incubated for 15 min at room temperature. The reactions were then initiated with the addition of 10 mL of DTNB, and 10 mL of either ACh or BCh. The hydrolysis of ACh or BCh was monitored by observing the formation of yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min. This anion forms as a result of the reaction between DTNB and thiocholine, and is released by the enzymatic hydrolysis of either ACh or BCh. All reactions were performed in triplicate in 96-well microplates using a VERSA max (Molecular Devices). The percentage (%) inhibition was calculated from (E–S)/E × 100, where E and S are the enzyme activities without and with the test sample, respectively. The ChEs-inhibiting activity of each sample was expressed in terms of the 50 % inhibition concentration (IC_{50}) value (mg/mL or mM required to inhibit the hydrolysis of the substrate; ACh or BCh, by 50 %), as calculated from the log-dose inhibition curve.

In vitro BACE1 enzyme assay

Each assay was carried out according to the supplied instructions with selected modifications. Briefly, mixtures of 10 µL of assay buffer (50 mM sodium acetate, pH 4.5), 10 µL of BACE1 (1.0 U/mL), 10 µL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM, ammonium bicarbonate), and 10 µL of samples (final concentration, 250 µg/mL for the extracts/fractions; 100 µM for the compounds) dissolved in 10 % DMSO were incubated for 60 min at 25 C in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by BACE1 was monitored by formation of the fluorescent donor Rh-EVNL (530-545 nm, excitation; 570-590 nm, emission), the abundance of which was determined by measuring the increase in fluorescence excited at 545 nm and recorded at 585 nm. Fluorescence was measured with a microplate spectrofluorometer (Molecular Devices). The percent inhibition (%) was obtained by the following equation: % Inhibition = $[1 - (S_{60} - S_0)/(C_{60} - C_0)] \times 100$, where C_{60} was the fluorescence of the control (enzyme, buffer, and substrate) after incubation for 60 min, C_0 was the initial fluorescence of the control, S_{60} was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after

incubation for 60 min, and S_0 was the initial fluorescence of the tested samples. To account for the quenching effect of samples, the sample solution was added to a separate reaction mixture C, and any reduction in fluorescence by the sample was investigated. The BACE1 inhibitory activity of compounds was expressed in terms of the IC_{50} value (µg/mL or µM required to inhibit proteolysis of the substrate, BACE1, by 50 %), as calculated from the log-dose inhibition curve. Quercetin was used as a positive control.

Assay for $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was assessed by the modified method of Kooy et al. [22], which involved monitoring highly fluorescent rhodamine 123 that was rapidly produced from non-fluorescent DHR 123 in the presence of $ONOO^-$. In brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5.0 mM potassium chloride, and 100 µM DTPA. The final DHR 123 concentration was 5.0 µM. The assay buffer was prepared prior to use and placed on ice. The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic $ONOO^-$ (10 µM) dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a fluorescence microplate reader (Bio-Tek Instruments Inc., FL 9 800, Winooski, UT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. The values of the $ONOO^-$ scavenging activity were calculated as the final fluorescence intensity minus the background fluorescence, via detection of DHR 123 oxidation. L-Penicillamine was used as the positive control.

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.

RESULTS:

AChE, BChE and BACE1 inhibitory activities of the EtOH extract and its solvent soluble fractions from *O. sanctum*

To evaluate the anti-AD potential of *O. sanctum*, the EtOH extract was tested in *in vitro* AChE, BChE, and BACE1 inhibition assays. The inhibitory activities of the EtOH extract against AChE, BChE, and BACE1 are shown in Table 1. The EtOH extract showed potential inhibitory activity against AChE, BChE, and BACE1 with IC_{50} values of 11.42 ± 0.23, 93.04 ± 0.43, and 90.43 ± 1.73 µg/mL compared to the positive controls of Berberine for ChEs (0.13 ± 0.01 µg/mL for AChE, and 8.28 ± 0.22 µg/mL for BChE) and Quercetin for BACE1 (10.7 ± 0.17 µg/mL). The EtOH extract of *O. sanctum* was dissolved in H_2O and successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH to obtain different solvent-soluble fractions, which were individually tested for AChE, BChE, and BACE1 inhibitory activity. As shown in Table 1, the CH_2Cl_2 and EtOAc fractions showed the highest AChE inhibitory activity with IC_{50} values of 2.54 ± 0.03 and 13.26 ± 0.44 µg/mL, whereas the CH_2Cl_2 and EtOAc fractions also showed the potential BChE inhibitory activity with IC_{50} values of 13.52 ± 0.13 and 19.56 ± 0.34 µg/mL respectively. Moreover, the CH_2Cl_2 fraction exhibited the highest BACE1 inhibitory activity with an IC_{50} value of 3.05 ± 0.01 µg/mL.

Table 1: AChE, BChE and BACE1 inhibitory activities of the ethanolic extract and its solvent-soluble fractions from *O. sanctum*

Extract/Fractions	IC_{50} values (µg/mL)[a] ± SEM		
	AChE	BChE	BACE1
EtOH extract	11.42 ± 0.23	93.04 ± 0.43	90.43 ± 1.73
CH_2Cl_2 fraction	2.54 ± 0.03	13.52 ± 0.13	3.05 ± 0.01
EtOAc fraction	13.26 ± 0.44	19.56 ± 0.34	16.98 ± 0.27
n-BuOH fraction	36.86 ± 0.70	42.94 ± 0.70	46.17 ± 0.47
H_2O fraction	49.81 ± 1.21	NA	NA
Berberine[b]	0.13 ± 0.01	8.28 ± 0.22	
Quercetin[c]			10.7 ± 0.17

[a] The concentration that caused 50% inhibition (IC_{50}) is given as the mean ± SEM of triplicate experiments
[b] Used as positive control in AChE and BChE inhibitory assay
[c] Used as positive control in BACE1 inhibitory assay
NA no activity in tested concentration

Table 2: Peroxynitrite ($ONOO^-$) scavenging activities of the ethanolic extract and its solvent-soluble fractions from *O. Sanctum*

Extract/Fractions	IC_{50} values (µg/mL)[a] ± SEM
	Peroxynitrite ($ONOO^-$)
EtOH extract	8.81 ± 0.18
CH_2Cl_2 fraction	1.21 ± 0.05
EtOAc fraction	7.46 ± 0.16
n-BuOH fraction	35.15 ± 0.23
H_2O fraction	NA
L-Penicillamine[b]	1.38 ± 0.08

[a] The concentration that caused 50% inhibition (IC_{50}) is given as the mean ± SEM of triplicate experiments
[b] Used as positive control in $ONOO^-$ scavenging assay
NA no activity in tested concentration

Table 3: Antioxidant and cholinesterase inhibitory activities of compounds isolated from *O. Sanctum*

Compounds	IC_{50} (µM) ± SEM[a]			
	$ONOO^-$	AChE	BChE	BACE1
Cirsilineol	2.85 ± 0.04	2.95 ± 0.02	3.25 ± 0.08	20.35 ± 0.21
Isothymusin	5.49 ± 0.20	8.25 ± 0.13	7.85 ± 0.01	4.45 ± 0.05
L-Penicillamine[b]	9.25 ± 0.61			
Berberine[c]		0.70 ± 0.21	11.64 ± 0.81	
Quercetine[d]				9.85 ± 0.39

[a] The concentration that caused 50% inhibition (IC_{50}) is given as the mean ± SEM of triplicate experiments
[b] Used as positive control in $ONOO^-$ scavenging assay
[c] Used as positive control in AChE and BChE inhibitory assay
[d] Used as positive control in BACE1 inhibitory assay

Peroxynitrite ($ONOO^-$) scavenging activity of the EtOH extract as well as different fractions from *O. sanctum*

Peroxynitrite ($ONOO^-$) scavenging activities of the EtOH extract and its different solvent-soluble fractions of *O. sanctum* are presented in Table 2. CH_2Cl_2 fraction displaying the highest $ONOO^-$ inhibitory activity with an IC_{50} value of 1.21 ± 0.05 µg/mL compared to the positive control L-Penicillamine with an IC_{50} value of 1.38 ± 0.08 µg/mL. In addition, the EtOAc and *n*-BuOH fractions also showed significance inhibitory activity with corresponding IC_{50} values of 7.46 ± 0.16 and 35.15 ± 0.23 µg/mL, respectively, results were shown in table 2.

Antioxidant and cholinesterase inhibitory activities of compound isolated from *O. sanctum*

The inhibitory activities of the isolated compounds against AChE, BChE, BACE1 and $ONOO^-$ are expressed as IC_{50} values in Table 3. Among the tested compounds, Cirsilineol showed the most potent inhibitory activity against AChE and BChE with an IC_{50} value of 2.95 ± 0.02 µM and 3.25 ± 0.08 µM, whereas berberine had an IC_{50} value of 0.70 ± 0.21 µM for AChE and 11.64 ± 0.81 µM for BChE. Isothymusin also showed significant AChE as well as BChE inhibitory activity, with IC_{50} values of 8.25 ± 0.13 µM and 7.85 ± 0.01 µM, respectively. Furthermore, Isothymusin displayed potent concentration-dependent inhibitors of BACE1, with IC_{50} values of 4.45 ± 0.05 µM, compared to the positive control quercetin, which had an IC_{50} value of 9.85 ± 0.39 µM. Cirsilineol also showed significance inhibitory activity against BACE1, with IC_{50} values of 20.35 ± 0.21 µM. In addition, both compounds exhibited significant inhibitory effects against $ONOO^-$, with scavenging potencies as indicated by IC_{50} values of 2.85 ± 0.04, and 5.49 ± 0.20 µM, respectively whereas IC_{50} values of the positive controls, L-penicillamine was 9.25 ± 0.61 µM, results were shown in table 3.

DISCUSSION:

In 2015, 46.8 million people worldwide are living with AD. This number of AD patients will dramatically increase over every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2030 [23]. AD is one of the most costly diseases to cure in developed countries [24]. This cholinergic change is believed to represent the earliest determined neurochemical event leading to AD [25]. Two major hypotheses have been proposed regarding the molecular mechanism of the pathogenesis of AD: the cholinergic hypothesis and the amyloid cascade hypothesis. In order to treat and prevent AD, most pharmacological research has focused on AChE and BChE inhibitors to alleviate the cholinergic deficit and to improve

neurotransmission [26]. AChE is the main enzyme responsible for the hydrolysis of ACh at the cholinergic synapse, while BChE acts as a co-regulator of the activity of AChE. Under normal physiological conditions, maximum ChEs activity is due to AChE. However, as the disease progress, AChE activity decreases in specific brain regions, whereas BChE activity increases, compensating for some of the functions of AChE in cholinergic neurons. Consequently, therapeutic agents that serve as inhibitors of both these enzymes could provide additional benefits in AD [27]. Moreover, considerable evidence from genetics and molecular biology supports the "amyloid-cascade hypothesis," which states that Aβ production and excessive accumulation are the principal pathogenetic events leading to AD [28]. BACE1 is the rate-limiting enzyme in the proteolytic processing of APP and is required for the production of Aβ. BACE1 levels and activities are increased in AD [29,30]. Furthermore, BACE1 which is involved in the first and rate-limiting step of Aβ formation from APP, has also generated great interest and several BACE1 inhibitors are currently being studied in clinical trials [31]. AD continues to be described as highly connected with cellular oxidative stress, which includes augmentation of protein oxidation, protein nitration, glycol oxidation, and lipid oxidation together with the accumulation of Aβ [32,33]. Among cellular oxidative stress, $ONOO^-$, have been associated with the etiology of several human degenerative disorders. Particularly, $ONOO^-$, produced by the in vivo reaction of nitric oxide (NO) with O_2^-, has been implicated in Aβ formation and accumulation, with high levels of Aβ also augmenting $ONOO^-$ generation in the brain of AD patients [34].

Ocimum sanctum L. (Lamiaceae), a well-known herbal medicine, is widely distributed throughout the world [13]. 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]. *O. sanctum* shows ameliorative potential in attenuating vincristine induced peripheral neuropathic pain in rats, which may be attributed to decrease in oxidative stress and calcium levels. Administration of OS (100 and 200 mg/kg p.o.) and its saponin rich fraction (100 and 200 mg/kg p.o.) for 14 days significantly attenuated vincristine-induced neuropathic pain along with decrease in oxidative stress and calcium levels [20]. Giridharan et al. reported that *O. sanctum* Linn. Leaf extracts inhibit acetylcholinesterase and improve cognition in rats with experimentally induced dementia. *O. sanctum* treatment significantly increased the induration in the DNCB skin test. Therefore, *O. sanctum* was shown to be useful for the management of experimentally induced cognitive dysfunctions in rats [39]. In Morris water maze test, OS pretreatment improves reference memory, working memory and spatial learning. Both ibotenic acid and colchicine induced deficits in active avoidance learning and retention of learned behavior were significantly reversed. OS might be effective in clinical Alzheimer's disease by virtue of its cognition enhancement, antidepressant and antianxiety properties, which are primary needs to be addressed in alzheimer's disease [40]. In our present study, we found that ethanol extract of *O. sanctum* showed potent inhibitory activities against AChE, BChE, BACE1, and antioxidant scavenging activity. Among the tested fractions, CH_2Cl_2 fraction showed potential activity compared to other polar fractions. The CH_2Cl_2 fraction was found as the most active fraction by AChE, BChE and BACE1 inhibitory assays. The CH_2Cl_2 fraction was also shown to possess strong inhibitory activities against peroxynitrite ($ONOO^-$) scavenging assay. Considering the inhibitory potential, CH_2Cl_2 fraction was selected for chromatographic separation in order to determine the active compounds from *O. sanctum*. Repeated chromatography of the CH_2Cl_2 fraction yielded two flavonoid compounds, Cirsilineol and isothymusin, was found to be the most active compound in the AChE, BChE and BACE1 inhibitory assays as well as $ONOO^-$ inhibitory assay. Therefore, our results clearly demonstrated that *O. sanctum* and its constituent have great value in the development of therapeutic and preventing agents for AD. Further *in vivo* and cell-based studies are needed to clarify the detailed mechanism of action of these compounds in the brain membrane and other organs.

CONCLUSION:

The present bioactivity-guided fractionation and isolation study of *O. sanctum* on BACE1 and ChEs inhibition demonstrated that the isolated constituents exerted potential BACE1 along with ChEs inhibitory effects, and $ONOO^-$ scavenging effect, suggesting their potential role for treating AD. In particular, the AChE, BChE, BACE1 along with $ONOO^-$ inhibitory activities of the isolated compounds were 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 AD by alleviating oxidative stress

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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