Neuroprotective Effects of *Ocimum basilicum* Extract against Hydrogen Peroxide-Induced Oxidative Stress in SK-N-SH Neuroblastoma Cells
(Kesan Neuromelindungi Ekstrak *Ocimum basilicum* terhadap Tekanan Oksidatif yang Diaruh Hidrogen Peroksida dalam Sel Neuroblastoma SK-N-SH)

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's and Parkinson's disease are characterized by the progressive loss of neurons. One of the contributing factors for these diseases is oxidative stress, characterized by the imbalance of free radicals production and antioxidant defense mechanisms. In the present study, the neuroprotective effects of Ocimum basilicum *var.* thyrsiflora *against hydrogen peroxide (H_2O_2)-induced oxidative stress in SK-N-SH neuroblastoma cells were evaluated. The exposure of SK-N-SH cells to 50 µM H_2O_2 for 24 h induced cytotoxicity and apoptosis as measured by cell viability and flow cytometry, respectively. Pretreatment with ethyl acetate (ObEA) fraction at 3.1-25 µg/mL showed the highest protection against H_2O_2-induced cell death compared to other fractions and crude extract by increasing cell viability and reducing apoptosis. The evaluation of antioxidant capacity via 1, 1-diphenyl-2-picrylahydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assays showed ObEA possessed the highest antioxidative properties. The intracellular reactive oxygen species (ROS) production of H_2O_2 in untreated cells increased by 2.39-fold compared to the control and was significantly attenuated by the 2 h pre-treatment of* O. basilicum *(p<0.05). The reduction in intracellular superoxide dismutase (SOD) induced by H_2O_2 was also abrogated by the pretreatment of* O. basilicum. *These findings suggested that* O. basilicum *is potentially neuroprotective against oxidative damage in neuronal cells by scavenging free radicals, restoring SOD activities and eventually prevent cell death.*

Keywords: Antioxidant; neuroprotective; Ocimum basilicum*; rosmarinic acid*

ABSTRAK

Penyakit kemerosotan saraf seperti Alzheimer dan Parkinson dicirikan oleh kehilangan neuron secara progresif. Salah satu faktor penyumbang penyakit ini ialah tekanan oksidatif, yang dicirikan oleh ketidakseimbangan antara penghasilan radikal bebas dan mekanisme pertahanan antioksidan. Dalam kajian ini, kesan neuromelindungi Ocimum basilicum *var.* thyrsiflora *terhadap tekanan oksidatif yang diaruh hidrogen peroksida (H_2O_2) dalam sel neuroblastoma SK-N-SH telah dinilai. Pendedahan sel SK-N-SH terhadap 50 µM H_2O_2 selama 24 jam telah menyebabkan sitotoksik dan apoptosis yang masing-masing diukur melalui kebolehhidupan sel dan aliran sitometri. Pra-rawatan dengan fraksi etil asetat (ObEA) pada 3.1-25 µg/mL menunjukkan kesan perlindungan tertinggi terhadap kematian sel yang diaruh H_2O_2 berbanding fraksi lain dan ekstrak mentah dengan meningkatkan kebolehhidupan sel dan mengurangkan apoptosis. Penilaian kapasiti antioksidan melalui asai difenil-pikrilhidrazil (DPPH) dan asai kuasa antioksidan penurunan ferik (FRAP) menunjukkan ObEA mempunyai ciri antioksidan tertinggi. Pengeluaran spesies oksigen reaktif (ROS) intrasel dalam sel yang hanya dirawat H_2O_2 meningkat sebanyak 3.29 kali ganda berbanding dengan sel kawalan dan dilemahkan secara signifikan oleh 2 jam pra-rawatan* O. basilicum *(p<0.05). Pengurangan superoksida dismutase (SOD) intrasel yang disebabkan oleh H_2O_2 juga turut dilemahkan oleh pra-rawatan* O. basilicum. *Keputusan kajian ini mencadangkan bahawa* O. basilicum *berpotensi berfungsi sebagai agen neuromelindungi terhadap kerosakan oksidatif dalam sel-sel neuron dengan merencatkan radikal bebas, memulihkan aktiviti SOD dan akhirnya menghalang kematian sel neuron.*

Kata kunci: Antioksidan; asid rosmarinik; neuromelindungi; Ocimum basilicum

INTRODUCTION

Neurodegenerative disease is characterized by the progressive loss of neurons. One of the known causes for the disease is oxidative stress due to the imbalance between free radical production and antioxidant defence systems. Reactive oxygen species (ROS) induce loss of cellular membrane integrity and some protein functions including superoxide dismutase (SOD) (Avery 2011). Furthermore, neurons are susceptible to the free radicals due to high consumption of oxygen (Steinbrenner & Sies 2013). Besides having synthetic drugs, the potential of natural sources as neuroprotective agent for neurodegenerative

diseases has gained huge attention among researchers. For instance, plants with potent antioxidant activities such as *Centella asiatica*, *Gingko biloba* and *Panax ginseng* have been explored for their neuroprotective potential (Kumar & Khanum 2012).

Ocimum basilicum or commonly known as sweet basil belongs to the Lamiaceae family. Modern pharmacological studies have reported some therapeutic potential showed by *O. basilicum* such as antioxidant (Kaurinovic et al. 2011; Kwee & Niemeyer 2011; Patil et al. 2011; Rameshrad et al. 2015), antiinflammatory (Rameshrad et al. 2015), anticancer (Kathirvel & Ravi 2012), antimicrobial (Srivastava et al. 2014a) and neuroprotective activities (Bora et al. 2011; Koutroumanidou et al. 2013). Rosmarinic acid is reported as the major bioactive compound in *O. basilicum* which possesses antioxidant properties (Jayasinghe et al. 2003; Srivastava et al. 2014b). However, the majority of antioxidative capacity of *O. basilicum* has been previously reported based on the direct interaction of the plant/oil extract with chemical reagents such as in DPPH and FRAP assays and not via bioassay studies. In addition, there is only a limited number of studies which have been documented on the antioxidant and neuroprotective effects of the *O. basilicum* extract compared to the other *Ocimum* species i.e. *Ocimum sanctum*.

O. basilicum var. *thyrsiflora* is one of the *O. basilicum* cultivars and it is known as Thai basil. It has been widely used in Asian culinary and traditional remedies. To the extent of our knowledge, there has been no reported *in vitro* study on this plant for the neuroprotective effects particularly via antioxidant mechanism. Thus, this study aimed to evaluate the neuroprotective effects of *O. basilicum* var. *thyrsiflora* against oxidative stress induced by H_2O_2 in SK-N-SH neuroblastoma cells. SK-N-SH cell is a suitable cell model to be used for neuroprotective studies as it shows neuronal phenotype with expression of numerous neurochemical markers (Jayaraj et al. 2013).

Materials and Methods

Preparation of Plant Materials

Ocimum basilicum var. *thyrsiflora* plants were obtained from local plantation in Gombak, Kuala Lumpur and a voucher specimen (HF100) was deposited at the Herbarium UKM Bangi. The leaves were dried and extracted with 80% ethanol at room temperature for 3 days and then filtered using Whatman No 1 filter paper. The extraction and filtration were repeated for 3 times. The filtrate was collected and concentrated under reduced pressure using a rotary evaporator. The extract was freeze-dried to get the crude extract (ObCE) and stored at 4°C until further used. To prepare the fractions of *O. basilicum*, ObCE was macerated and sonicated in hexane solvent for 3 h at each cycle and filtered using Whatman No 1 filter paper. The extraction was repeated until clear solution was obtained showing that no more non-polar compounds being extracted. The filtrate was collected and concentrated

under reduced pressure using a rotary evaporator to obtain the hexane fraction (ObHex). ObHex was stored at 4°C until further used. Meanwhile, the residue from ObHex was subsequently extracted with ethyl acetate solvent by using the same procedure to obtain the ethyl acetate fraction (ObEA). The last residue was known as ethanol fraction (OBEtOH).

Determination of Total Phenolic Content Using Folin-Ciocalteu Method (FC)

Total phenolic content was determined using FC method adapted to 96-well plate as described by Zongo et al. (2010) with minor modifications. Briefly, 100 µL of FC reagent (10% v/v) was added into 20 µL of samples (ObCE, ObHex, ObEA and ObEtOH) dissolved in DMSO (100 µg/mL). After 5 min incubation, 80 µL of sodium carbonate (75 g/L) was added to each well. The 96-well plate was slightly shaken and incubated for 30 min at room temperature in the darkness. The absorbance was measured at 735 nm using microplate reader. Gallic acid was used as standard and total phenolic content was expressed as gallic acid equivalent per gram of sample based on equation of gallic acid calibration curve Y = 0.0047X (R^2 = 0.9992) where Y is the absorbance values and X is the gallic acid concentration.

Quantification of Rosmarinic Acid Content in *O. basilicum* by HPLC Analysis

Chromatographic analysis was performed using High Performance Liquid Chromatography Prominence Shimadzu (HPLC Shimadzu LC-20AT) equipped with quaternary pump, diode array detector (DAD), autosampler, thermostated column oven, degasser and LC Solutions software. Separations were carried out on a SunFire C18 (5 µm, 4.6 × 50 mm) column.

The separation was conducted according to Srivastava et al. (2014). In summary, deionised water + 0.1% ortho-phosporic acid and methanol (HPLC grade) + 0.1% ortho-phosporic acid were used as mobile phase A and mobile phase B, respectively, by following the gradient program as follows: 0-2 min, 0% B (isocratic), 2-5 min, 40% B (linear gradient), 5-10 min, 50% B (linear gradient), 10-18 min, 50% B (isocratic), 18-23 min, 40% B (decreasing gradient), 23-25 min, 0% B (equilibration). The flow rate of the elution was 1.0 mL/min. The column was maintained at 25°C throughout the analysis. The detection wavelength was set at 280 nm with an injection volume of 20 µL. Linear standard calibration curve of rosmarinic acid (0.2-1.0 mg/mL) were plotted to obtain unknown concentration of rosmarinic acid in *O. basilicum*. The validation of HPLC was carried out by determination of linearity, precision, limit of quantification (LOQ), and limit of detection (LOD).

DPPH Radical Scavenging Activity Assay

The determination of antioxidant activity of *O. basilicum* and its major phenolic compound, rosmarinic acid were

carried out using DPPH scavenging assay adapted to 96-well plate described by Zongo et al. (2010) with minor modifications. Graded concentrations of samples were prepared ranging from 1.953 to 1000 µg/mL obtained by two-fold dilutions. Briefly, 100 µL of DPPH solution (10% dissolved in DMSO) was added to the 100 µL of samples and the mixture was allowed to stand at room temperature in the dark for 60 min. The absorbance was read at 540 nm using microplate reader. Gallic acid and quercetin were used as standard reference. The scavenging activity of DPPH radical was calculated as:

$$\%RSA = [1-(A_1 - A_2) / A_0] \times 100$$

where A_0 is the absorbance of control (without sample); A_1 is the absorbance of sample; and A_2: Absorbance of sample (without DPPH radical).

The results were expressed as IC_{50} value (concentration at which 50% inhibition of DPPH radical) which were obtained from the relationship curves of scavenging activities (%) versus linear range of sample concentrations. The antioxidant activity index (AAI) was measured as; AAI = [DPPH] (µg/mL)/ IC_{50} (µg/mL) in which [DPPH] is the final concentration of DPPH radicals.

FERRIC REDUCING/ANTIOXIDANT POWER (FRAP) ASSAY

FRAP assay was conducted according to Yang et al. (2011) with minor modifications. All solutions were freshly prepared. Briefly, 270 µL of FRAP reagent consisting of 300 mM acetate buffer (pH3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (in 40 mM HCl) and 20 mM ferric chloride at 10:1:1 (v/v/v) was added to the 30 µL of sample (1 mg/mL dissolved in DMSO) and warmed at 37°C for 4 min. Gallic acid and quercetin were used as standard reference. The absorbance was read at 593 nm. Different concentrations of trolox (0.195 to 200 µg/mL) were used to plot standard curve and results obtained were expressed as FRAP value (mg trolox/ mg sample dry weight).

CELL CULTURE AND TREATMENT

SK-N-SH neuroblastoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Eagle Minimum Essential Medium (EMEM; Gibco, USA) enriched with 10% fetal bovine serum (FBS; Tico Europe) and 1% penicillin-streptomycin (Nacalai Tesque, Japan). Cells were grown in 75 cm² tissue culture flask and kept at 37°C in 5% CO_2. The medium was changed every 2 days and cells were passaged once they reached 80% confluence.

MTT CELL VIABILITY ASSAY

The effect of the sample treatment on SK-N-SH cells viability was measured using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. SK-N-SH cells were plated (5×10^4 cells/well) into 96-well plate and incubated overnight as described previously. Cells were treated with *O. basilicum*, rosmarinic acid (Sigma, USA) and N-Acetyl-L-cysteine (NAC; Sigma, USA). NAC is a potent antioxidant to combat oxidative stress and has been demonstrated to display neuroprotective activities (Nazıroğlu et al. 2014). After treatment, MTT reagent (5 mg/mL) was added to each well and incubated for 4 h at 37°C. Then, culture medium containing MTT was discarded and DMSO was added to solubilize the purple formazan. After 15 min of incubation, the absorbance was measured at 517 nm using ELISA microplate reader (Tecan, Switzerland). Cell viability was expressed as the percentage relative to untreated cells.

APOPTOSIS ASSAY BY ANNEXIN V-FITC/PI STAINING

The mode of cell death induced by H_2O_2 was assessed by flow cytometry using Annexin V-FITC/PI assay (BD Bioscience, USA). SK-N-SH cells were pretreated with various concentration of ObCE, ObEA, ObEtOH, rosmarinic acid and NAC for 2 h prior to 50 µM H_2O_2 for 24 h. After treatment, cells were harvested then washed with chilled PBS before suspended in Annexin binding buffer and incubated with fluorescence isothiocyanate (FITC)-conjugated Annexin V 15 min and another 5 min with propidium iodide (PI). Then, 400 µL of ABB was added to the stained cells and the cell suspension was transferred to a polystyrene round-bottom tube prior to analysis with FACSCanto II flow cytometer.

INTRACELLULAR ROS MEASUREMENT

Intracellular ROS level was assessed by dichloro-dihydro-fluorescein diacetate (DCFH-DA) labeling assay as previously described by Chan et al. (2010). Treated cells were harvested and washed with chilled PBS. The cell pellet was suspended in 1 mL pre-warmed serum-free medium and then 1 µl of 10 mM DCFH-DA (Life Technologies, USA) was added into the suspension. Staining was performed in dark at 37°C followed by centrifugation at 220×g for 5 min. The cells were washed with 1 mL chilled PBS (2 times) and the supernatant was discarded. Afterwards, 500 µL of chilled PBS was added to resuspend the pellets. The stained cell suspension was transferred to polystyrene round-bottom tube and analyzed using BD FACSCanto II Flow Cytometer.

INTRACELLULAR SUPEROXIDE DISMUTASE (SOD) ACTIVITY ASSESSMENT

The intracellular SOD activity was measured spectrophotometrically according to the instruction given in the SOD assay kit (Abcam, USA). Briefly, treated cells were harvested and then washed with chilled PBS at 250×g (4°C) for 10 min and supernatant was discarded. Then, cell pellets were sonicated in chilled PBS and spun at 1500×g for 10 min. Supernatant were collected and then spinned at 10,000×g for 15 min. Later, the supernatant was collected and used to measure SOD activity at 450 nm by using microplate reader (Tecan, Switzerland).

STATISTICAL ANALYSIS

All data are presented as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by post-Dunnett's analysis using GraphPad Prism 5 Software was performed and values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

TOTAL PHENOLIC CONTENT

ObEtOH contained the highest polyphenol content which was 125.80 mg/g gallic acid equivalent, followed by ObEA (100.97 mg/g GAE), ObCE (88.32 mg/g GAE) and ObHex (50.61 mg/g GAE) which may account for the observed biological effects in the present study. However, the total phenolic contents among the crude extract and fractions were not statistically different. Previously, Bora et al. (2011) also have reported that *O. basilicum* contains phenolic, flavonoids and tannins which demonstrated neuroprotective effects in ischemia and reperfusion-induced cerebral damage in mice.

HPLC ANALYSIS

HPLC chromatogram of *O. basilicum* exhibited the peak of rosmarinic acid corresponding to the retention time at 15.01±0.02 min (Figure 1). The quantification of rosmarinic acid in the *O. basilicum* showed ObEtOH contained the highest amount of rosmarinic acid (37.81±1.57 µg/mL) followed by ObCE (37.02±1.65 µg/mL), ObEA (33.66±0.75 µg/mL) and ObHex (9.61±0.21 µg/mL). It can be suggested that the extraction yield (rosmarinic acid) increases with increasing polarity of the solvent used in extraction. It also can be found that the content of rosmarinic acid in ObEtOH is slightly high than the crude extract (ObCE). This result indicates that increasing polarity of the solvent in sequential fractionation enhances rosmarinic acid concentration. The calibration curve plotted for the rosmarinic acid standard over the concentration range of 200-1000 µg/mL showed a correlation coefficient (R^2) of 0.995. The relative standard deviations (% RSD) were measured to confirm the reproducibility of the results. The % RSD values for

intra-day of the mean retention time and mean area under the peak were 0.358% and 3.256%, respectively, whereas the % RSD values for inter-day of the mean retention time and mean area under the peak were 0.274% and 3.652%, respectively. The LOD and LOQ of rosmarinic acid were found to be 2.258 and 6.843 µg/mL, respectively.

Our HPLC analysis confirmed the presence of rosmarinic acid compound which has been reported as the major bioactive compound in *O. basilicum* (Lee 2010; Srivastava et al. 2014; Zgorka et al. 2001). In accordance with the highest total phenolic content, ObEtOH also showed the highest rosmarinic acid concentration compared to the crude extract and other fractions.

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF *O. BASILICUM*

In this study, we evaluated the antioxidant capacities of *O. basilicum* using DPPH and FRAP assays, the two widely used assays for evaluation of *in vitro* antioxidant activities (Alam et al. 2013). The hydroethanolic crude extract, fractions of *O. basilicum* and rosmarinic acid were tested for their free radical scavenging activity on DPPH using quercetin and gallic acid as standards. Based on the radical scavenging activity (% RSA) regression curve obtained (data not shown), the IC_{50} for standards and samples were identified as 50% of inhibition concentration. ObEA showed free radical scavenging activity with the AAI of 0.32 (Table 1). Interestingly, rosmarinic acid showed efficient scavenging activity compared to the standard quercetin while gallic acid standard showed the highest AAI. However, the IC_{50} of ObCE, ObHex and ObEtOH cannot be determined.

The FRAP values ranged from 0.67±0.06 to 2.48±0.01 mg/mg (Table 1). Gallic acid showed the strongest antioxidant consistent with its highest antioxidant abilities in DPPH assay while ObEA showed the highest FRAP value compared to the crude extract and other fractions.

In both DPPH and FRAP assays, ObEA showed the highest antioxidant capacity compared to the crude extract and other fractions. This result showed that high phenolic content did not necessarily result in potent antioxidant activities. Our findings are in agreement with previous studies of Bhebhe et al. (2015), Chew et al. (2011) and Kasparavičienė et al. (2013) who reported that no
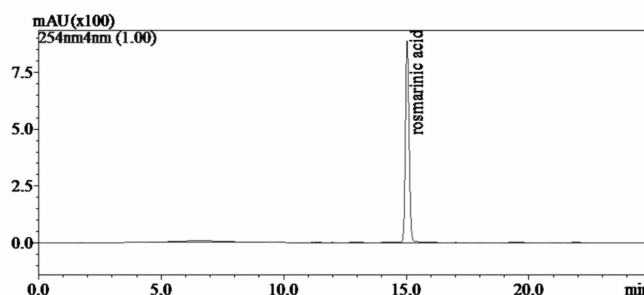

FIGURE 1. HPLC chromatogram of rosmarinic acid standard; the peak at retention time of 15.01±0.02 min

TABLE 1. Antioxidant activity of *O. basilicum* and rosmarinic acid.
Data are presented as mean ± SEM (*n*=3)

Sample	DPPH		FRAP
	IC$_{50}$	AAI	Equivalent trolox amount (mg/mg)
Quercetin	23.87±5.17	2.09	2.46±0.01
Gallic acid	2.48±0.01	13.32	2.48±0.01
Rosmarinic acid	17.04±1.31	2.93	2.21±0.03
ObCE	nd	nd	1.24±0.03
ObHex	nd	nd	0.67±0.06
ObEA	156.74±6.93	0.32	1.36±0.02
ObEtOH	nd	nd	1.28±0.05

nd: not detectable

correlation has been noted between phenolic contents and antioxidant capacity. In contrast, a study conducted by Sultana et al. (2009) showed plants with higher total phenolic content exhibited greater antioxidant activity.

PROTECTIVE EFFECTS OF *O. BASILICUM* ON H$_2$O$_2$-INDUCED NEURONAL DEATH

High phenolic fractions of *O. basilicum* (ObEA, ObEtOH) and crude extract (ObCE) were subjected for the *in vitro* study. The results showed that H$_2$O$_2$ reduced SK-N-SH cell viability in a dose-dependent manner after 24 h incubation (Figure 2(A)). It is well documented that H$_2$O$_2$ is widely used to induce oxidative stress in SK-N-SH cells (Choi et al. 2013; Ezoulin et al. 2008; Hu et al. 2012). At 50 µM of H$_2$O$_2$, cell viability was reduced to 60.9% as compared to the control group. This concentration was therefore used in this study to induce oxidative stress. Pretreatment with ObCE, ObEA, ObEtOH, rosmarinic acid and NAC 2 h prior
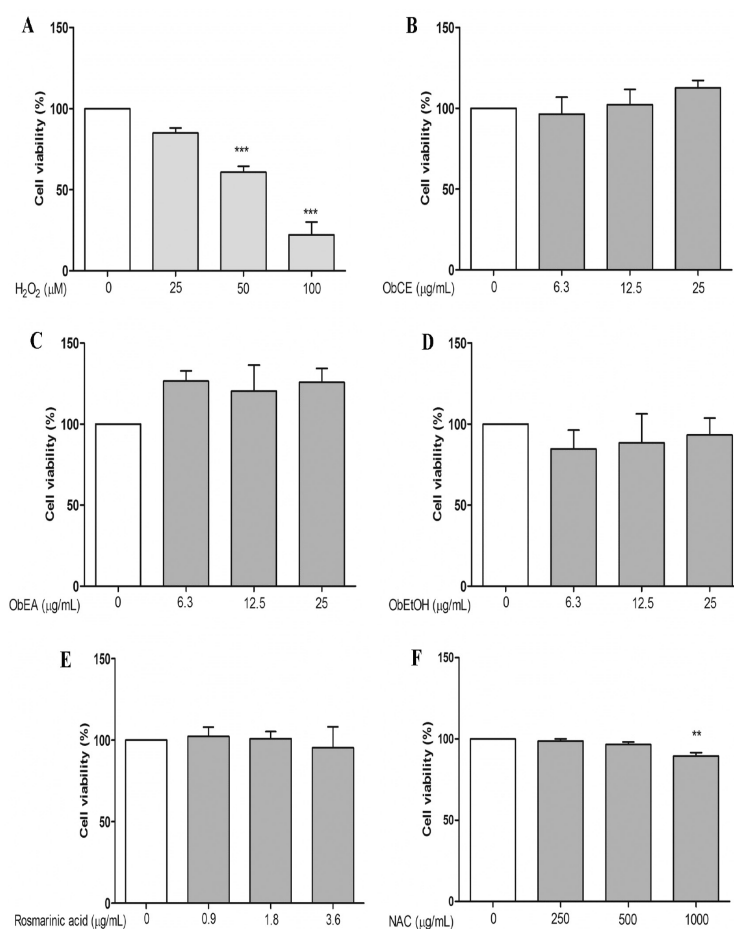
Data are presented as mean ± SEM (*n*=3). **p<0.01, ***p<0.001 vs. control group

FIGURE 2. (A) Effect of H$_2$O$_2$ on SK-N-SH cell viability. Treatment with *O. basilicum* (B) ObCE, (C) ObEA, (D) ObEtOH, (E) rosmarinic acid and (F) NAC did not cause cytotoxicity in SK-N-SH cells at all concentrations

to H_2O_2-induced cytotoxicity were found to attenuate the effects of H_2O_2 and significantly increased cell viability (Figure 3). At all the tested concentrations, ObCE, ObEA, ObEtOH, rosmarinic acid and NAC alone did not show any obvious toxicity on the viability of SK-N-SH cells (Figure 2(B-F)).

### EFFECTS OF *O. BASILICUM* ON H_2O_2-INDUCED APOPTOSIS IN SK-N-SH CELLS

The protective effects of *O. basilicum*, rosmarinic acid and NAC against H_2O_2-induced apoptosis were measured by flow cytometry. After exposure to H_2O_2 only for 24 h, cells showed higher apoptotic event compared to necrotic event (Figure 4). The percentage of viable cells, apoptotic and necrotic cells at 50 μM H_2O_2 were $23.8 \pm 3.6\%$, $65.9 \pm 9.9\%$ and $10.4 \pm 6.8\%$, respectively. The percentage of viable and apoptotic cells showed a significant difference ($p<0.05$) compared to the control but not the percentage of necrotic cells. Our results were in agreement with previous studies which reported that H_2O_2 induced apoptosis in several cell lines (Chen et al. 2016; Jin et al. 2013; Yu et al. 2016). Cells pretreated with ObEA and NAC for 2 h prior to the

H_2O_2 induction increased cell viability in dose-dependent manners. Pretreatment of cells with ObCE also showed a dose-dependent protective actions against H_2O_2-induced apoptosis up to 12.5 μg/mL. However, cell viability of the cells pretreated with ObEtOH and rosmarinic acid did not increase.

It is interesting to note that the percentage of viable cells detected in Annexin V staining was lower compared to the MTT result. In fact, vast literature has been published on the effect of several phytochemicals and plant extracts on MTT assay which potentially reduced MTT in the absence of cells (Peng et al. 2005; Shoemaker et al. 2004). Similarly, Chan et al. (2006) has reported that there was a discrepancy between MTT results and acridine orange/propidium iodide (AO/PI) staining which suggested possible compound interaction with MTT.

EFFECTS OF *O. BASILICUM* ON H_2O_2-INDUCED PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS)

Intracellular ROS production was detected with 2,7'-dichlorofluorescein (DCF) diacetate. Cells treated with 50 μM H_2O_2 for 24 h showed a significant increase
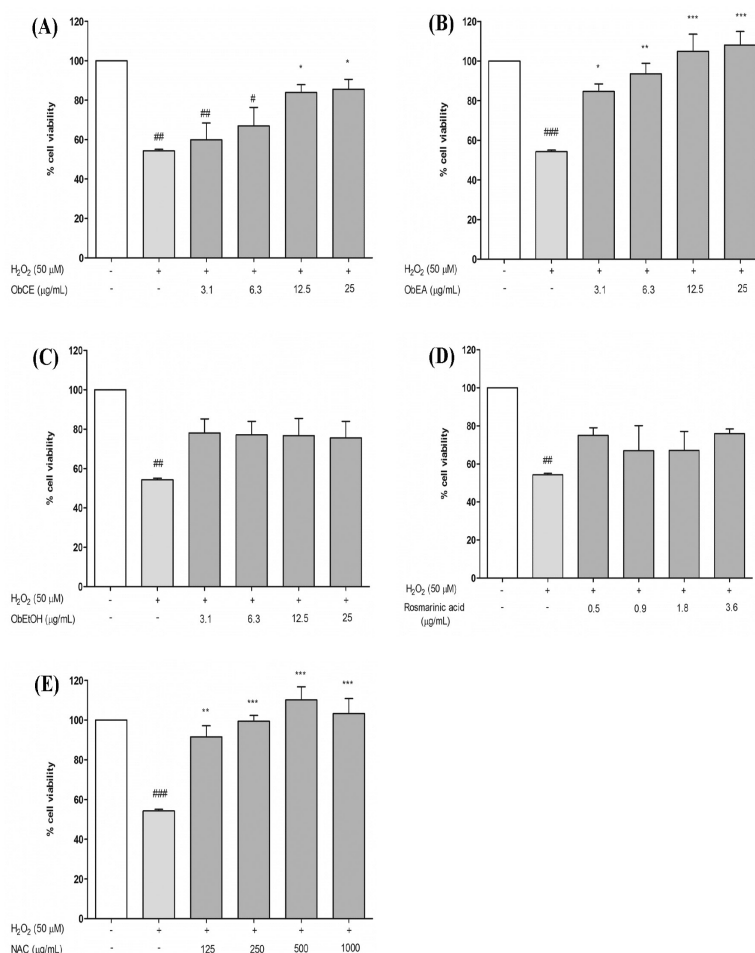
Data are presented as mean ± SEM ($n=3$). #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs. control, *$p<0.05$ **$p<0.01$, ***$p<0.001$ vs. H_2O_2 untreated group

FIGURE 3. The protective effects of (A) ObCE, (B) ObEA, (C) ObEtOH, (D) rosmarinic acid and (E) NAC with indicated concentrations on H_2O_2-induced cytotoxicity in SK-N-SH cells as determined using MTT assay
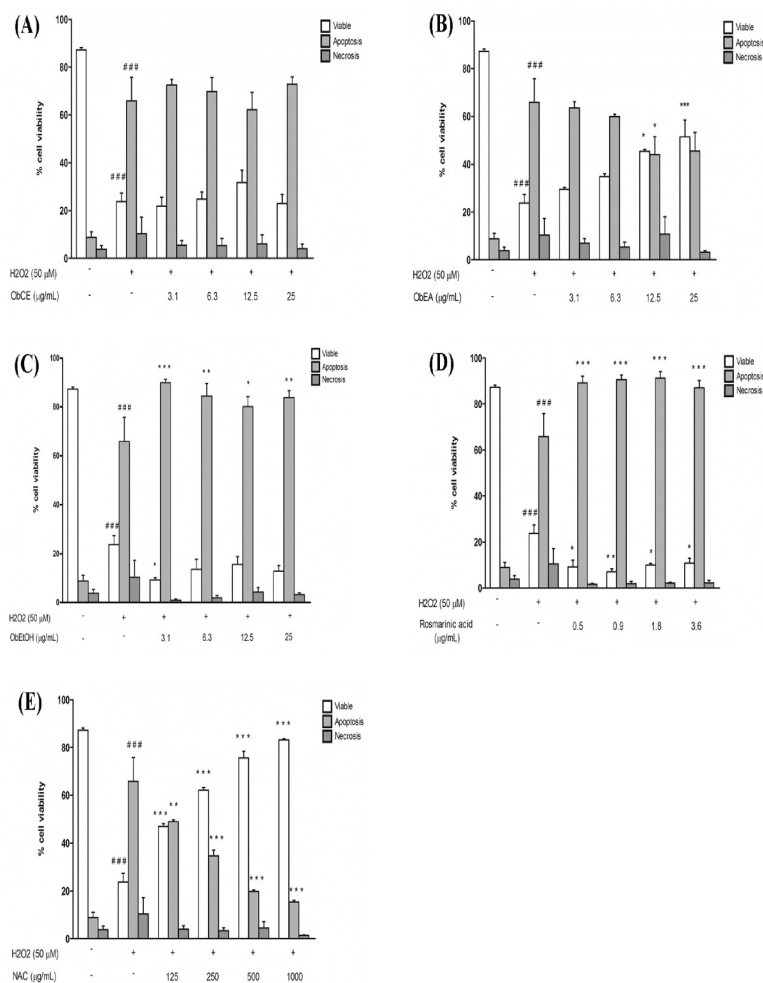
Data are presented as mean ± SEM (n=3). ###p<0.001 vs. control, *p<0.05 **p<0.01, ***p<0.001 vs. H_2O_2 untreated group

FIGURE 4. The protective effects of (A) ObCE, (B) ObEA, (C) ObEtOH, (D) rosmarinic acid and (E) NAC with indicated concentrations on H_2O_2-induced cytotoxicity in SK-N-SH cells as determined using Annexin V-FITC/PI

in ROS levels 2.39-fold compared to control (p<0.001) (Figure 5). However, cells pretreated with ObCE, ObEA, ObEtOH, rosmarinic acid and NAC (Figure 6(A)-6(E)) significantly reduced the ROS levels compared to cells induced with H_2O_2 alone. Increase in ROS generation caused the imbalance redox state in cell and initiated the oxidative stress. Pretreatment with *O. basilicum* significantly reduced the elevated ROS levels caused by H_2O_2 treatment. Similar results were observed when cells were pretreated with rosmarinic acid and NAC. Pre-treatment of cells with lower concentrations of *O. basilicum* was enough to reduce the ROS accumulation approaching the control values. The inhibition of ROS accumulation by *O. basilicum* appears to protect the SK-N-SH cells from H_2O_2-induced cytotoxicity. These findings demonstrate that *O. basilicum* is a potent antioxidant, which might be due to ROS scavenging property of *O. basilicum* and eventually contribute to its neuroprotective effect. A previous study indicated that *O. basilicum* extract possessed protective effects on ischemia and reperfusion-induced cerebral damage and motor dysfunctions in mice through restoration of oxidative stress

marker; glutathione (GSH) (Bora et al. 2011). Our data confirm that *O. basilicum* has antioxidant activity against H_2O_2-mediated intracellular ROS accumulation.

EFFECTS OF *O. BASILICUM*, ROSMARINIC ACID AND NAC ON SOD ACTIVITY IN SK-N-SH CELLS INDUCED WITH H_2O_2

Exposure of SK-N-SH cells to 50 μM H_2O_2 for 24 h reduced intracellular SOD level from 0.42±0.04 U/mL to 0.32±0.02 U/mL. The H_2O_2-induced SOD depletion in SK-N-SH cells has been previously demonstrated in a few studies (Li et al. 2014; Zhang et al. 2017). Excessive superoxide can decrease the antioxidant enzyme activities which explain the reduction of SOD level in our study. This effect was mitigated by pretreatment with ObCE, ObEA, ObEtOH, rosmarinic acid and NAC (Figure 7(A)-7(E)). At the lowest concentration, pre-treatment of cells with ObEtOH prior to H_2O_2-induced SOD depletion showed the highest SOD activity showing that the optimal concentration of ObEtOH to trigger the SOD activity was 3.1 μg/mL.
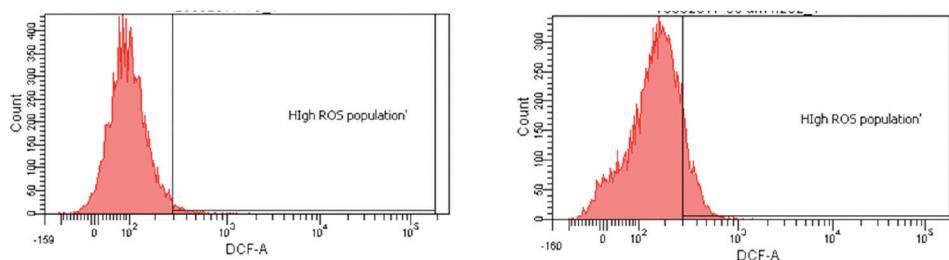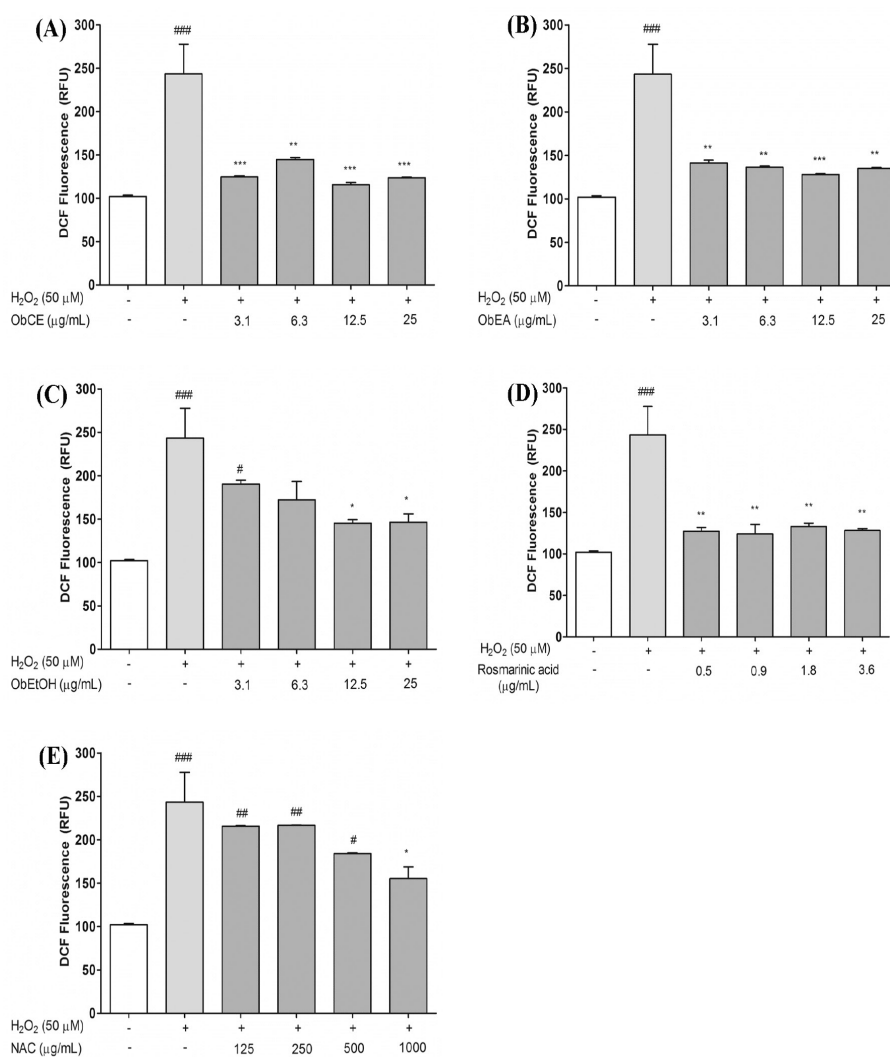
FIGURE 5. The chromatogram of DCF fluorescence (A) Untreated SK-N-SH cells,
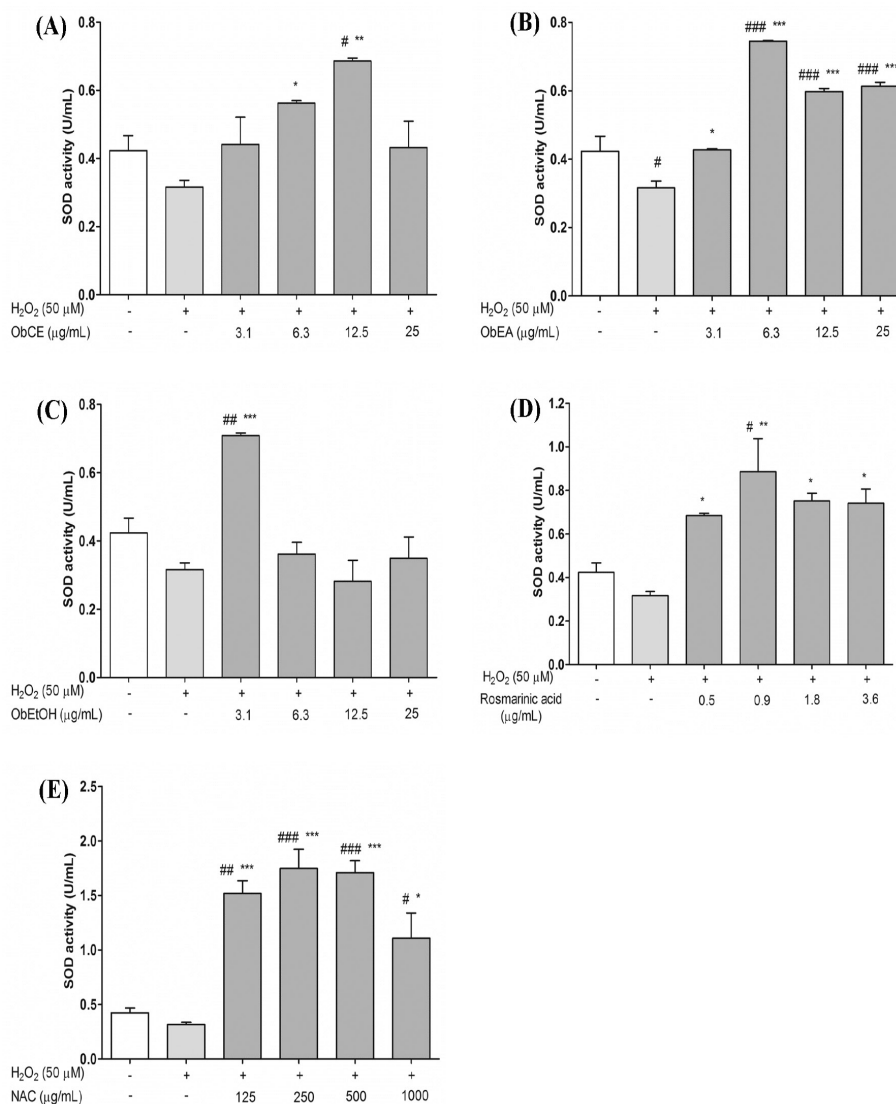(B) SK-N-SH cells treated with 50 μM H$_2$O$_2$ after 24 h

Data are presented as mean ± SEM (*n*=3). #*p*<0.05. ##*p*<0.01, ###*p*<0.001 vs. control, **p*<0.05 ***p*<0.01, ****p*<0.001 vs. H$_2$O$_2$ untreated group

FIGURE 6. The protective effects of (A) ObCE, (B) ObEA, (C) ObEtOH, (D) rosmarinic acid and
(E) NAC on H$_2$O$_2$-induced ROS production in SK-N-SH cells

Nevertheless, the SOD levels were higher than the control group, suggesting that *O. basilicum* up-regulated intracellular SOD levels. SOD is an endogenous antioxidant enzyme which helps to eliminate free radicals (Reiter 1995) in maintaining optimal cellular functions. SOD is known as the first line of defence which is responsible to dismutate superoxide radicals into hydrogen peroxide (less reactive species) which is then further converted to water and oxygen by catalase (Li et al. 2016). Therefore, the observed effects with *O. basilicum* are suggestive of neuroprotection by ways of enhancing the activity of endogenous antioxidant enzyme.

Data are presented as mean ± SEM (n=3). $^{\#}p<0.05$. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. control, $^{*}p<0.05$ $^{**}p<0.01$, $^{***}p<0.001$ vs. H_2O_2 untreated group

FIGURE 7. The activity of SOD of (A) ObCE, (B) ObEA, (C) ObEtOH, (D) rosmarinic acid and
(E) NAC on H_2O_2-induced in SK-N-SH cells

CONCLUSION

Our study showed that at molecular level, *O. basilicum* exerted neuroprotection against H_2O_2-induced cytotoxicity by lowering oxidative damage characterised by the reduction of intracellular ROS generation and restoration of intracellular SOD levels. Taken collectively, *O. basilicum* may directly act as a radical scavenger to eliminate ROS and eventually enhance intracellular SOD activity. These properties may explain its neuroprotective effects observed in our study which aided in protecting neuronal cells from H_2O_2-induced oxidative damage and could be potentially explored as a prevention modality of neurodegenerative diseases.

ACKNOWLEDGEMENTS

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