

# Protective effects of ethyl acetate extract of *Eclipta prostrata* against 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells

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**ABSTRACT:** Oxidative stress plays an important role in the pathogenesis of Parkinson's disease. The neurotoxin 6-hydroxydopamine initiates cellular oxidative stress which has been implicated in neurodegenerative conditions related to Parkinson's disease. *Eclipta prostrata*, also known as false daisy, is a plant belonging to the sunflower family. It is a rich source of phenolic compounds that confer the plant an antioxidant activity. In this study, we investigated the protective effects of ethyl acetate extract of *E. prostrata* against 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells. Pretreatment with the ethyl acetate extract of *E. prostrata* before exposing the cells to 6-hydroxydopamine significantly increased cell survival and reduced intracellular reactive oxygen species formation in a concentration-dependent manner. In addition, the ethyl acetate extract of *E. prostrata* suppressed the Bax/Bcl-2 ratio and reduced caspase-3 activity, a key modulator of cell apoptosis. Our finding clearly shows that the ethyl acetate extract of *E. prostrata* possesses a potential antioxidant capacity and attenuates SH-SY5Y cell death by blocking apoptotic signalling caused by 6-hydroxydopamine. *E. prostrata* could therefore be used as a potential herb to prevent oxidative stress-induced neurodegeneration in Parkinson's disease.

**KEYWORDS:** Bax/Bcl-2 ratio, caspase-3

## INTRODUCTION

Parkinson's disease (PD) is a movement disorder characterized by a complex and progressive neurodegeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in the depletion of dopamine (DA). The disease affects about 1–2% of the world population over 60 years of age<sup>1</sup>. The prevalence of PD is approximately 0.24% of Thai population. It is particularly common in areas of pesticide use<sup>2</sup> and it is expected to rise significantly. The etiology of PD has not been fully elucidated, but it is accepted that oxidative stress may be a major cause of the neurodegeneration. DA metabolism has been reported to play a pivotal role in the pathogenesis of PD<sup>3</sup>. Excess amount

of DA can either undergo auto-oxidation or enzymatic metabolism into toxic DA-quinone species and various active free radicals<sup>4,5</sup>, which causes mitochondrial dysfunction leading to cell degeneration. In addition, the study of postmortem brain tissues of PD patients revealed a marked increase in malondialdehyde<sup>6</sup>, lipid, and cholesterol hydroperoxide<sup>7</sup> as well as protein carbonyls<sup>8</sup>, supporting the occurrence of oxidative stress in the brain.

The neurotoxin 6-hydroxydopamine (6-OHDA) damages dopaminergic neurons by two mechanisms: formation of highly active free radicals<sup>9,10</sup> and disruption of mitochondria respiratory chain function<sup>11</sup>. Thus it is capable of inducing the neuropathological damage observed in PD both in vivo<sup>12</sup> and in vitro models<sup>13</sup>.

*Eclipta prostrata*, also known as ‘false daisy’, or ‘ka-meng’ in Thai, is a plant that belongs to the Asteraceae family. It is widely distributed throughout China, India, and Thailand. It has long been used as a traditional medicine for health remedies against atherosclerosis, diabetes mellitus, and hepatic disorders<sup>14–16</sup>. It exhibits a diversity of pharmacological capacities including antioxidant, anti-inflammatory, hypolipidaemic, hypoglycaemic, analgesic, antibacterial, antifungal, and antiviral<sup>17</sup>. Phytochemical constituents of *E. prostrata* consist mainly of alkaloids, glycosides, coumestans, triterpenes, flavonoids, and sterols<sup>18</sup>. Flavonoids act in this plant as natural antioxidants<sup>19</sup>. Previous studies revealed that a butanol fraction of the whole plant could increase the level of acetylcholine and attenuate oxidative stress in the brain and serum of rats, suggesting its use to prevent memory impairment<sup>20</sup>. A hydroalcoholic extract obtained from the aerial parts markedly ameliorates ischaemia-induced neuronal loss of the rat brain<sup>21</sup>. Although *E. prostrata* has been reported to possess neuropharmacological profile, its neuroprotective effects against oxidative stress-induced pathogenesis of PD have not been evaluated. Thus the present study determines the antioxidant activity and protective effects of *E. prostrata* against 6-OHDA-induced neurotoxicity in SH-SY5Y cells.

## MATERIALS AND METHODS

### Plant material

The ethyl acetate extract of *E. prostrata* was prepared according to a previous publication<sup>22</sup>. The dried powder (aerial parts) of *E. prostrata* was sequentially extracted with hexane, chloroform, and ethyl acetate (EtOAc). The filtrate of each solvent was evaporated to dryness in vacuo to give the hexane, chloroform, and EtOAc extracts, respectively. The EtOAc extract was used in this study. The EtOAc extract was dissolved in DMSO (Sigma-Aldrich) at 100 mg/ml as a stock solution and the final concentration of DMSO used in all experiments was 0.1% in the cell culture medium.

### Total phenolic and flavonoid contents

The phenolic content of the EtOAc extract was evaluated using Folin-Ciocalteu reagent (Merck Millipore)<sup>23</sup>. Thirty  $\mu$ l of the EtOAc extract (5 mg/ml) was dissolved in distilled water (2.37 ml), mixed with 150  $\mu$ l of freshly prepared diluted Folin Ciocalteu reagent, and incubated at room temperature for 1 min. Then the samples were added with saturated

$\text{Na}_2\text{CO}_3$  solution (450  $\mu$ l) and further incubated for 40–60 min in the dark. The samples were measured at 765 nm by using a UV-Vis-spectrophotometer (Jasco Corporation). Gallic acid was used as a standard and the total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of EtOAc extract.

In addition, flavonoid quantification of the EtOAc extract was performed using colorimetric method with modifications<sup>24</sup>. Briefly, 300  $\mu$ l of the EtOAc extract (5 mg/ml) dissolved in distilled water (1680  $\mu$ l) was mixed with 60  $\mu$ l of potassium acetate 1 M, 900  $\mu$ l of 95% ethanol, and 60  $\mu$ l of 10%  $\text{AlCl}_3$ . After incubation in the dark for 30 min, the absorbance of the mixture was measured at 415 nm using a UV-Vis-spectrophotometer (Jasco Corporation). The flavonoid content was expressed as mg quercetin equivalents (QE)/g of EtOAc extract.

### Cell culture

Human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection. The characteristics of SH-SY5Y cells have been described by Xie et al<sup>25</sup>. Cells were maintained at 5%  $\text{CO}_2$ , 37°C in 1:1 mixture, Dulbecco’s Modified Eagle’s medium (DMEM)/Ham-F12 supplemented with 10% heat inactivated foetal bovine serum, 25 mg/ml of penicillin, 25 U/ml of streptomycin, and 1 mM nonessential amino acids. After seeding, cells were grown for 24 h to reach 80–90% confluence.

### Cell viability assay

Neuronal cell survival was evaluated using resazurin assay (Sigma-Aldrich). Resazurin, a nonfluorescent dye, is converted to the highly red fluorescent dye resorufin by viable cells. Briefly, cells were seeded on 96-well culture plates at a density of  $4 \times 10^4$  cells/well and left for 24 h. Then, the cells were incubated with different concentrations of the EtOAc extract (1, 5, 10, 20, 40, 80, and 100  $\mu$ g/ml) of *E. prostrata* for 24 h or preincubated with 5, 10, and 20  $\mu$ g/ml of the EtOAc extract for 1 h followed by exposure to 50  $\mu$ M 6-OHDA for 24 h. The 6-OHDA (10 mM stock solution in 0.01% ascorbic acid; Sigma-Aldrich) was diluted in medium to obtain a concentration used in every experiment. After treatment, the resazurin solution (10% of cell culture volume) was added to the well plate and further incubated at 37°C and 5%  $\text{CO}_2$  for 2–3 h in the dark. The fluorescence was measured at 530 and 590 nm on a microplate reader (Bio-Tek Instruments). The percentage of cell viability

was expressed relative to the fluorescence of the corresponding control.

### ROS assay

The reagent 2',7'-dichlorodihydrofluorescein diacetate ( $H_2DCFDA$ ) is widely used to evaluate oxidative reaction and free radical generation in the cells. The interaction of DCFH with various oxidants has been reviewed<sup>26</sup>. ROS production was quantified by using this fluorescent probe according to the manufacturer's protocol (Invitrogen) with modifications. Cells were preincubated with 5, 10, and 20  $\mu g/ml$  of the EtOAc extract for 1 h before exposure to 6-OHDA (50  $\mu M$ ) for 24 h. After treatment, the medium was removed and the cells were then stained with 20  $\mu M$  of DCFH-DA for 30 min at 37 °C and 5%  $CO_2$  in the dark followed by washing two times with phosphate buffered saline and adding phenol-red free DMEM. The cells were analysed immediately by a fluorescence microplate reader (Bio-Tek Instruments) at 485 and 528 nm. The intracellular ROS levels are proportional to the DCF fluorescent intensity. The data are reported as fold change from the control condition.

### Western blots

To further investigate the protective effects of the EtOAc extract against 6-OHDA-induced neuronal apoptosis, the ratio between Bax and Bcl-2 expression was determined using western blot. After treatment under identical condition as described above, the cells were collected and lysed in ice-cold RIPA buffer with protease inhibitor cocktails (Sigma-Aldrich). The lysates were centrifuged at 1300g and 4 °C for 15 min. Protein concentrations were determined by Bradford protein assay (Bio-Rad). Equal amounts of proteins (20  $\mu g$ ) were separated by using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary polyclonal rabbit anti-Bax and rabbit anti-Bcl-2 (1:1000 dilution; Cell Signalling Technology). Primary monoclonal mouse anti-GAPDH (1:2500 dilution; Thermo Scientific Pierce) was used as internal control. After incubation with specific HRP-conjugated anti-IgG antibody (1:10 000 dilution; Invitrogen), immunoreactive bands were visualized using luminata forte western HRP substrate (Merck Millipore) and chemiluminescence imaging system (Syngene). The band densities of the interested proteins were normalized to that of GAPDH.

### Caspase-3 fluorometric assay

The protective effect of the EtOAc extract against 6-OHDA-triggered apoptosis was determined by using fluorescent caspase-3-like activity assay (Biovision). After treatment, all samples were centrifuged at 1300g, 4 °C for 15 min. The pellets were re-suspended in 50  $\mu l$  chilled cell lysis buffer and incubated for 10 min on ice. Then, the samples were added 50  $\mu l$  of 2  $\times$  Reaction Buffer (containing 10 mM DTT) and further added 5  $\mu l$  of 1 mM DEVD-AFC substrate (50  $\mu M$  final concentration), followed by incubation at 37 °C and 5%  $CO_2$  for 2 h in the dark. The cleavage of DEVD-AFC substrate was measured at 400 and 505 nm by using a fluorescence microplate reader (Bio-Tek Instruments).

### Statistical analysis

The results were expressed as mean  $\pm$  SEM of four to six independent experiments in which triplicate samples were performed. Statistical significance was assessed by using one-way ANOVA followed by a Tukey comparison test in GRAPHPAD PRISM 5. A value of  $p < 0.05$  was considered statistically significant.

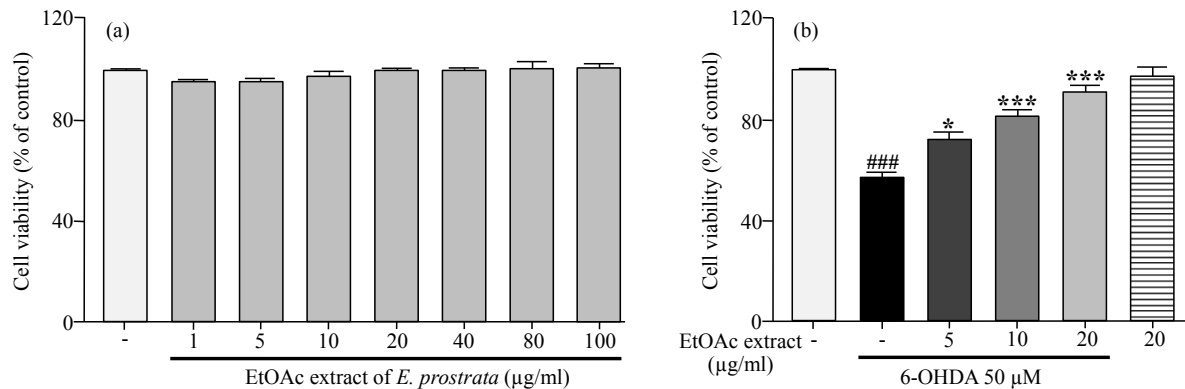
## RESULTS

### Analysis of total phenolic and flavonoid contents

The amount of total phenolic content was calculated from the regression standard curve equation:  $y = 1.0253x - 0.0194$ ,  $r^2 = 0.9994$ , while the flavonoid contents of the extract was calculated from the standard curve equation:  $y = 0.0064x - 0.0123$ ,  $r^2 = 0.9988$ . The total phenolic and flavonoid contents presented in the EtOAc extract of *E. prostrata* were found to be  $32.0 \pm 1.3$  mg GAE/g EtOAc extract and  $56.9 \pm 1.4$  mg QE/g EtOAc extract, respectively.

### Protective effect of the extract on 6-OHDA-induced SH-SY5Y cytotoxicity

When neuronal cells were incubated with various concentrations of the EtOAc extract (1–100  $\mu g/ml$ ), the EtOAc extract did not affect cell viability at any of the concentration tested (Fig. 1a). Thus the EtOAc extract was not toxic to the cells. Preincubation with the EtOAc extract (5, 10, and 20  $\mu g/ml$ ) for 1 h, prior to 6-OHDA (50  $\mu M$ ) treatment for 24, showed that the EtOAc extract could increase the cell viability to  $71.1 \pm 4.0$ ,  $80.5 \pm 3.6$ , and  $90.5 \pm 2.7\%$ , respectively, while incubation with 6-OHDA alone reduced the level of cell survival to  $56.6 \pm 2.2\%$  (Fig. 1b).



**Fig. 1** Neuroprotective effects of the EtOAc extract of *E. prostrata* on 6-OHDA-induced cytotoxicity in SH-SY5Y cells. The cells were incubated with various concentrations of the extract (1–100 µg/ml) for 24 h (a), or pretreated with the extract at 5, 10, and 20 µg/ml for 1 h, followed by exposure to 6-OHDA (50 µM) for 24 h (b). The percentage of cell viability was reported as mean ± SEM of four (a) and six (b) separated experiments performed in triplicate. ###  $p < 0.001$  as compared with the untreated control; \*  $p < 0.05$  and \*\*\*  $p < 0.001$  as compared with 6-OHDA treatment alone.

#### Effect of the extract on 6-OHDA-induced free radical formation

In 6-OHDA-treated cell alone, 6-OHDA markedly increased the intracellular ROS level to  $1.55 \pm 0.10$  fold of control, whereas preincubation with 5, 10, and 20 µg/ml of the EtOAc extract could significantly decrease the levels of intracellular ROS to  $1.17 \pm 0.03$ ,  $0.95 \pm 0.05$ , and  $0.84 \pm 0.03$  fold of control as compared with the 6-OHDA-treated group, respectively (Fig. 2).

#### Protective effect of the extract on 6-OHDA-induced the level of Bax and Bcl-2 expression

As shown in Fig. 3, it was found that the expression of Bax protein was upregulated, while that of Bcl-2 protein was downregulated in 6-OHDA treated group alone for 24 h. The Bax/Bcl-2 ratio was  $2.27 \pm 0.18$  fold of the control. Conversely, preincubation with the EtOAc extract at 5, 10, and 20 µg/ml could markedly suppress the level of Bax/Bcl-2 ratio to  $1.7 \pm 0.16$ ,  $1.17 \pm 0.15$ , and  $0.68 \pm 0.08$  fold of the control as compared with the 6-OHDA-treated group, respectively.

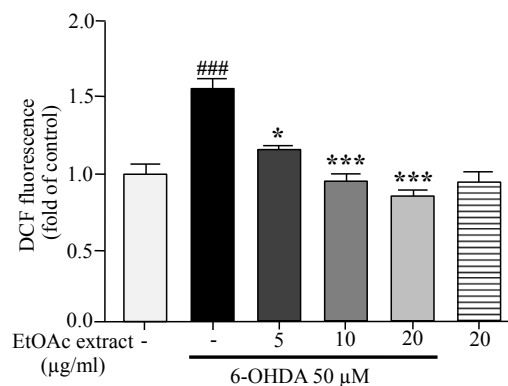
#### Effect of the extract on 6-OHDA-induced caspase-3 elevation

Preincubated cells with the EtOAc extract at 5, 10, and 20 µg/ml could significantly reduce caspase-3 activity to  $1.79 \pm 0.06$ ,  $1.54 \pm 0.06$ , and  $1.2 \pm 0.05$  fold as compared with the 6-OHDA-treated group, respectively. The treatment of 6-OHDA alone exhib-

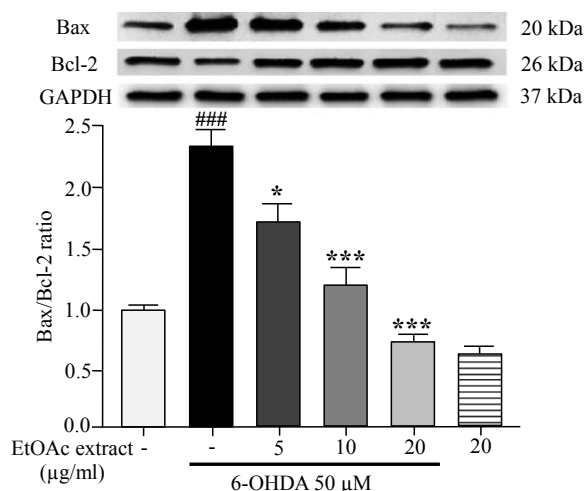
ited a remarkably high level of caspase-3 activity to  $2.0 \pm 0.11$  fold of the control (Fig. 4).

#### DISCUSSION

To date, herbal medicine are becoming an attracting alternative way to prevent several diseases, including PD since it is considerably safer than the conventional medications. Thus it seems necessary



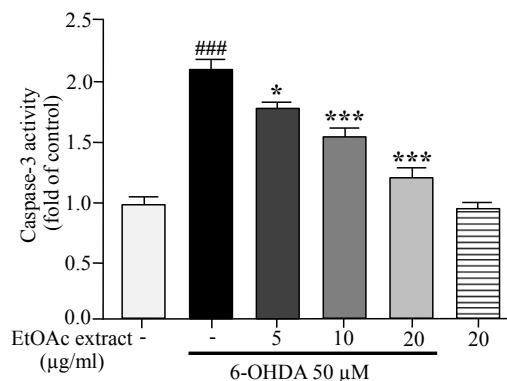
**Fig. 2** The antioxidant effect of the EtOAc extract of *E. prostrata* on 6-OHDA-induced generation of reactive oxygen species (ROS) in SH-SY5Y cells. The cells were exposed to 5, 10, and 20 µg/ml of the extract for 1 h, followed by exposure to 6-OHDA (50 µM) for 24 h. The ROS production was evaluated using  $H_2DCFDA$ , and the DCF fluorescence was expressed as fold of control. Results were reported as mean ± SEM of six individual experiments. ###  $p < 0.001$  as compared with the untreated control; \*  $p < 0.05$  and \*\*\*  $p < 0.001$  as compared with 6-OHDA treatment alone.



**Fig. 3** Effects of the EtOAc extract of *E. prostrata* on 6-OHDA-induced increase in Bax/Bcl-2 ratio. The cells were exposed to 5, 10, and 20 µg/ml of the extract for 1 h, followed by exposure to 6-OHDA (50 µM) for 24 h. After 24 h, the level of Bax/Bcl-2 was examined using western blot analysis. The GAPDH protein level was used as reference to control equal loading. Results were represented as mean ± SEM of six individual experiments. ###  $p < 0.001$  as compared with the untreated control; \*  $p < 0.05$  and \*\*\*  $p < 0.001$  as compared with 6-OHDA treatment alone.

to elucidate the beneficial effects of plant-derived flavonoid compounds for the development of novel herbal medicine. The plant-derived flavonoids that can suppress 6-OHDA-induced oxidative damage to neurons would be considered as a potential agent for preventing PD. We have investigated the mechanism by which the EtOAc extract of *E. prostrata* protects against 6-OHDA-induced SH-SY5Y cytotoxicity as an in vitro model of PD.

Oxidative stress mediated apoptosis in dopaminergic neurons is considered the major pathogenic cause of PD<sup>27</sup>. The augmentation of apoptotic downstream signalling was enhanced in response to 6-OHDA-derived cellular oxidative stress. Several studies reported that 6-OHDA could trigger the elevation of Bax/Bcl-2 ratio<sup>28,29</sup>. The ratio between pro-apoptotic protein Bax and antiapoptotic protein Bcl-2 is thought to play a role in apoptosis. Any shift in the balance of the Bax/Bcl-2 ratio would result in an increase in mitochondrial permeability followed by promoting the release of cytochrome c from mitochondria<sup>30</sup>, amplifying proteolytic caspase cascades and causing cell apoptosis. Caspase family has essential roles in cell apoptosis. There are two types



**Fig. 4** Effects of the EtOAc extract of *E. prostrata* on 6-OHDA-induced increase in caspase-3 activity. The cells were exposed to 5, 10, and 20 µg/ml of the extract for 1 h, followed by exposure to 6-OHDA (50 µM) for 24 h. The fluorescent intensity on caspase-3 activity was quantified using caspase-3 fluorometric assay kit and expressed as folds of the control. Results were represented as mean ± SEM from six individual experiments. ###  $p < 0.001$  as compared with the untreated control; \*  $p < 0.05$  and \*\*\*  $p < 0.001$  as compared with 6-OHDA treatment alone.

of apoptotic caspases: initiator caspases (caspases -2, -8, -9, and -10) and effector caspases (caspases -3, -6, and -7). Both initiator and effector caspases coordinately act together to trigger the final step of programmed cell death. Caspase-3 is activated after being triggered by the initiator caspases, resulting in a cellular commitment to death. 6-OHDA-induced neuronal cell death is dependent on the activation of caspase-3 activity<sup>31</sup>. Consistent with previous studies, our results found that 6-OHDA caused increase in intracellular ROS production, indicating a cellular oxidative stress. The oxidative stress-induced by 6-OHDA involved in the activation of apoptotic signalling, as evidence from the upregulation in the Bax/Bcl-2 ratio at protein level and the increase in caspase-3 activity which would potentially lead to neuronal cell death.

In the current study, we demonstrated that pretreatment with the EtOAc extract of *E. prostrata* suppressed 6-OHDA-induced intracellular ROS production in a concentration dependent manner. The EtOAc extract shows the highest free radical scavenging potency when compared to methanolic and chloroform extracts because of its high content of phenols and flavonoids<sup>32</sup>. Moreover, the EtOAc extract could attenuate 6-OHDA-induced neuronal apoptosis by decreasing the Bax/Bcl-2 ratio and the caspase-3 activity, suggesting that the protective ef-

fects of the EtOAc extract might be mainly due to its antioxidant property. Furthermore, previous studies showed that a hydroalcoholic extract of whole aerial parts of *E. prostrata* could prevent oxidative stress-induced ischaemic injury in rat brain by an elevation of antioxidant enzymes<sup>21</sup>. Another study reported that the ethanolic leaf extract expressed antioxidant capacity by augmenting catalase and glutathione activities, leading to a suppression of haloperidol-induced extrapyramidal movement disorders in the rats<sup>33</sup>. These results therefore confirm that phytochemical substances contained in *E. prostrata* are able to cross the blood-brain barrier and have a beneficial effect against oxidative stress-induced neuronal disorders, including PD.

In conclusion, this is the first report on the neuroprotective effects of *E. prostrata* against 6-OHDA induced-SH-SY5Y cell toxicity. The present study clearly demonstrated that *E. prostrata* exerts its antioxidant and antiapoptotic effects in part through the regulation of the expression of Bcl-2 family and caspase-3 activity. These findings therefore provide assurance that *E. prostrata* may have a potential as herbal medicine for the prevention of oxidative stress associated with neurodegenerative diseases such as PD.

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