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Original Article

# *In vitro* and *in vivo* evaluation of the neuroprotective activity of *Uncaria hirsuta* Haviland

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ABSTRACT

The incidence of neurodegeneration leading to the conditions such as Alzheimer's and Parkinson's diseases are on the increase, they require the approaches that focus on protection prevention rather than treatment. Plants are rich sources of many compounds which possess medicinal properties. We sought to investigate the neuroprotective effects of *Uncaria hirsuta* and its compounds on D-galactose-induced stress in BALB/c mice as well as 6-hydroxydopamine (6-OHDA)-induced stress in mouse nerve growth factor (mNGF)-differentiated PC12 cells. Our results demonstrate that the 95% ethanol extract of *U. hirsuta* reversed the D-galactose-induced learning and memory dysfunctions and decreased the malodialdehyde levels. Furthermore, the isolated compounds, 5$\beta$-carboxystrictosidine (**1**) and chlorogenic acid (**2**), protected mNGF-differentiated PC12 cells against toxicity induced by 6-OHDA by acting as antiapoptotic agents. The 50% inhibitory concentration ($IC_{50}$) for intracellular reactive oxygen species (ROS) scavenging was found to be 24.5 (for **1**) and 19.7 $\mu$M (for **2**), and both **1** and **2** reduced intracellular calcium levels with respective $IC_{50}$ values of 46.9 and 27 $\mu$M. Interestingly, both compounds inhibited caspase 3 and 9 activities with respective $IC_{50}$ values of 25.6 and 24.5 $\mu$M for **1** and 19.4 and 16.3 $\mu$M for **2**. Our results identify *U. hirsuta* and its active compounds as potential neuroprotective agents and deserve further evaluation for drug development for neuroprotection in the future.



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## 1. Introduction

Neurodegeneration is a progressive disorder or damage to neurons including their death. The term relates to any pathological condition primarily affecting neurons [1]. When monitored clinically, neurodegenerative diseases cover a large group of neurological disorders with heterogeneous clinical progression and pathological expression [2]. Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative diseases. These two disease display different clinical manifestations, however have some similar characteristic features, like the influence of genetic variants, environmental exposure, and behaviour. Many factors play key roles in disease onset, and neural loss is associated with pathogenic pathways like apoptosis, dysregulation of ion homeostasis, molecular damage, cell cycle disturbance, and metabolic changes [3,4]. Whereas there have been advancements in the science, there are still major unmet needs in the form of a lack of exact and appropriate information about how to prevent and treat these diseases. Drugs are available for AD and PD, but these drugs target the symptoms and not the cause. Therefore, identifying therapeutic intervention that can reverse or protect against these diseases is highly desirable.

Neurotoxins like botulinum neurotoxin and tetanus neurotoxin have specific patterns of entering neurons via interactions with gangliosides [5]. D-galactose is a neurotoxin for which chronic exposure to leads to memory loss, neurodegeneration, and oxidative damage [6,7]. Induced neurotoxicity using D-galactose leads to memory loss in animal models and rats fed D-galactose were found to develop neuropathy [8]. D-Galactose was found to shorten the lifespan in two species of flies; however its induction was associated with an increase in oxidative stress [9]. Oxidative stress leads to the lipid peroxidation, malondialdehyde (MDA) is one of the final products of the peroxidation [10]. On the other hand, 6-hydroxydopamine (6-OHDA) is a neurotoxin which selectively targets catecholaminergic neurons and is used for designing models for PD [7,11]. 6-OHDA is capable of causing neurotoxin-induced degeneration as well as several behavioural changes [12]. It can also impair mitochondrial function, induce oxidative stress and cause apoptosis [13,14]. PC12 is a cell line obtained from the pheochromocytoma of rat adrenal medulla, on the treatment with the nerve growth factor (NGF) they can differentiate in neuronal cell type [13]. They have been extensively used as the neuronal models for the study because of the features they exhibit are similar to the neuronal primary culture [15].

*Uncaria* is a genus very rich in medicinal values; species from the genus are crucial parts of traditional treatments in many countries, the plants from this genus are widely used traditionally to treat CNS related ailments. *Uncaria* is a genus very rich in medicinal values, found to be effective for asthma, rheumatism, hyperpyrexia, hypertension, headaches, cerebral haemorrhage, and neurodegenerative diseases. Isolated indole alkaloid compounds from the plants have high efficacy against hypertension, epilepsy, depression, AD, and PD [16,17]. Gastrodia and *Uncaria decoction* is traditional Chinese medicine (TCM) used to treat stroke in China [18]. Water extract *Uncaria rhynchophylla* is used as a TCM for hypertension and brain diseases [19]. It is also in TCM for treating cardiovascular and cerebrovascular diseases. However, TCM is not the only field where the medicinal properties of this genus have been explored, isolated indole alkaloid compounds from the genus *Uncaria* have high efficacy against hypertension, epilepsy, depression, AD, and PD [16]. Recent study found that *U. rhynchophylla* was found to alleviate the amyloid beta (Aβ) deposition and the associated neuropathology in five familial AD mutations (5XFAD) mice [20]. *Uncaria hirsuta* Haviland shows neuroprotective effects against toxicity induced by Aβ protein in cortical neurons of rats [21]. Genus *Uncaria* has shown its extraordinary therapeutic potential towards neurodegenerative diseases [16]. These facts lay a very strong foundation for neuroprotective effects of *U. hirsuta*. Thus, further investigations of *in vivo* and *in vitro* models are supported by the effects of the active compounds from *U. hirsuta* towards neuroprotection in very challenging neurodegenerative conditions.

## 2. Materials and methods

### 2.1. *Plant material and extract preparation of* U. hirsuta

Stems with hooks of *U. hirsuta* were collected from the Taiwan Seed Improvement and Propagation Station, Council of Agriculture (COA) (Hsinshe, Taichung, Taiwan) and identified by Mr. Chi-Luan Wen. A voucher specimen (M388) was deposited at the Graduate Institute of Pharmacognosy, Taipei Medical University (Taipei, Taiwan). Extraction was performed using collected plant material (dried stems with hooks, 1.5 kg) of *U. hirsuta* with 95% ethanol at 65 °C (UHE) or water at 100 °C (UHH) for 4 h, respectively; the process was repeated three times. The filtered solutions were combined and evaporated under reduced pressure to yield a total extract.

### 2.2. *Experimental animals and study protocol*

Eight-week-old male BALB/c mice ($n = 28$) were purchased from the National Laboratory Animal Centre (Taipei, Taiwan). Each mouse was individually housed in a wire-bottomed stainless steel cage in a temperature and humidity controlled room (at 22 °C) with a 12-h light/dark cycle. Mice had free access to standard mouse/rat chow (Prolab® RMH2500, 5P14 Diet; PMI Nutrition International, Brentwood, MO, USA) and water. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Taipei Medical University (LAC-99-0152). After acclimation for 1 week, mice were randomly divided into four groups ($n = 7$ in each group), including a blank group and three D-galactose-induced groups (one group with only D-galactose as control, two UH intervention groups, which includes UHH and UHE). Oxidative damage was induced using D-galactose, following a previously described procedure with modifications [22,23]. D-Galactose (12 g dissolved in 100 mL of normal saline) was subcutaneously injected once a day (injection titer, 0.1 mL per 10 g of mouse weight) into the dorsal neck of a BALB/c mouse for 8 weeks; in the meantime, groups of UHH and UHE (80 mg/kg/day) were orally administered

concurrent D-galactose subcutaneous injections for 8 weeks. In the blank group, mice were subcutaneously injected with normal saline concurrent with the oral administration of distilled water daily for 8 weeks. Plasma levels were determined at weeks 4 and 8. Mice in these four groups were trained and evaluated for learning dysfunction using a step-through passive avoidance test before the end of the experiments.

### 2.3. Learning behaviour evaluations by the step-through passive avoidance test

Mice learning behaviours were evaluated by step-through passive avoidance tests according to a previously described method with modification [24]. The test was performed during 3 successive days from days 49–51 using a ShuttleFlex (AccuScan Instruments, Columbus, Ohio, USA) apparatus, which included an illuminated chamber and a dark chamber separated by a guillotine door. The dark chamber was equipped with a metal floor that can provide electric foot shocks. The first day (a training day), a mouse was placed in the dimly lit room containing the ShuttleFlex apparatus for 30 min to allow it to acclimatize to the new environment. After that, the mouse was then placed in the illuminated chamber, facing away from the opened guillotine door and the dark chamber. Once the mouse entered the dark chamber, the guillotine door was immediately closed, and an electric shock (0.3 mA) was applied for 3 s from the metal floor. After 5 s in the dark chamber, the mouse was returned to the cage. If the mouse stayed in the illuminated chamber for up to 300 s, it was forced to enter the dark chamber (with the guillotine door closed), the same electric foot shocks were applied, and then it was returned to the cage. On the 3rd day, the formal test was run. The protocol of the formal test was the same as the first training except that the electric shock was not administered in the dark chamber. The retention test (step-through latency or the time staying in the illuminated chamber in seconds) was recorded, and the time limit for the mouse staying in the illuminated chamber was set to 300 s.

### 2.4. Blood and tissue treatment

During the experimental periods, blood samples of mice were collected by submandibular blood collection method at 4 and 8 weeks using 5-mm animal lancets. After the samples were centrifuged at 367 $\times g$, the plasma obtained was saved and stored at −80 °C for malondialdehyde (MDA) determination. At the end of the experiment, the mice were weighed and sacrificed, and blood samples were collected by cardiac puncture. The brains were isolated, and all samples were immediately stored at −80 °C for further measurements. For MDA contents in brain tissues, whole brains were immediately ground into a fine powder in liquid nitrogen in a mortar and pestle. The fine powder was suspended in 1 mL of 1X phosphate-buffered saline for protein extraction. After the suspensions were centrifuged at 12,500 $\times g$ at 0 °C for 60 min, the supernatants were stored at −80 °C for further investigation. The proteins were quantified using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a standard [25].

### 2.5. Oxidative status parameters

The MDA content in plasma (μM) or in organ extracts (μM/μg tissue) was determined by BIOXYTECH® MDA-586™ assay kits according to the manufacturer's instructions (Portland, OR, USA) based on the reaction of *N*-methyl-2-phenylindole with MDA to generate a chromogenic product with a maximum absorption at 586 nm, and results are expressed using 1,1,3,3-tetramethoxypropane as the standard [25].

### 2.6. Isolation, purification, and identification

The 95% ethanol extracts were sequentially fractionated with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH); three fractions EtOAc, *n*-BuOH and aqueous were obtained. Using bio-guided isolation, the *n*-BuOH fraction (10.0 g) was subjected to Diaion HP 20 column chromatography and eluted with an $H_2O$–MeOH gradient. Five fractions (UHE 1-1 to 1–5) (Chart S1) were obtained. UHE 1–3 was further separated using a C-18 column and eluted with 30%~100% MeOH; then three fractions (UHE1-3-1 to 1-3-3) were obtained. UHE1-3-1 was purified with a semi-preparative C-18 reverse-phase high-performance liquid chromatographic (HPLC) column (Biotic Aqu-ODS-W 5 μm, 10 mm × 250 mm; Biotic Chemical, Taipei, Taiwan) with 23% acetonitrile as the mobile phase, and compound **1** (22.5 mg) was obtained. UHE 1–2 was further separated with a C-18 column and eluted with 5% ~100% MeOH to obtain nine fractions (UHE1-2-1 to 1-2-9). UHE1-2-1 was purified with a semi-preparative C-18 reverse-phase HPLC column with 15% acetonitrile as the mobile phase, and compound **2** (5.2 mg) was obtained. UHE 1–5 was separated with MCI CHP-20P eluted with 30%~100% MeOH to obtain five fractions (UHE1-5-1 to 1-5-5). UHE1-5-1 was purified with a semi-preparative C-18 reverse-phase HPLC column with 30% acetonitrile as the mobile phase, and compound **3** (4.7 mg) was obtained. The structures of the purified compounds were identified by physical and spectral data ($^1$H-nuclear magnetic resonance (NMR) and $^{13}$C-NMR) with those in the literature.

### 2.7. Cell culture

PC12 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated horse serum (10%) and FBS (5%) at 37 °C in a humidified 5% $CO_2$ condition. PC12 cells were cultured for additional 5 days after seeding, and were then treated with mouse nerve growth factor (mNGF) and medium (1:100) for differentiation into neuronal-type cells.

### 2.8. Cell viability and neuroprotective analysis

After differentiation, mNGF-differentiated PC12 cells were treated with test samples for 6 h, and the cell viability was evaluated using the WST-8 assay [26]. To analyze the neuroprotective effect, mNGF-differentiated PC12 cells were pretreated with test samples for 6 h, followed by exposure to 6-OHDA for an additional 24 h. Cell viability was evaluated as described previously [26].

### 2.9. Anti-apoptosis analysis by flow cytometry using Annexin-V/propidium iodide (PI)

For the anti-apoptosis analysis, mNGF-differentiated PC12 cells were examined by Annexin-V/PI with flow cytometry. Briefly, cells were pretreated with test samples for 6 h, followed by exposure to 6-OHDA for an additional 24 h. The cell density was adjusted to $10^6$ cells/mL, and Annexin V-fluorescein isothiocyanate (FITC) and PI were added. The mixtures were incubated in the dark at room temperature for 15 min, then analyzed using an FACSCantoII flow cytometer (BD Biosciences, Brea, CA, USA) at an excitation wavelength of 488 nm [27].

### 2.10. Intracellular ROS analysis

mNGF-Differentiated PC12 cells were pretreated with test samples for 6 h, followed by exposure to 6-OHDA for an additional 24 h. ROS generation was analyzed with the intensity of the oxidized product, 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-$H_2$DCFDA) (with excitation at 485 nm and emission at 535 nm) using a microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA). Results are reported as multiples of the control [28].

### 2.11. Intracellular calcium ($Ca^{2+}$) analysis

mNGF-Differentiated PC12 cells were treated with the test compounds for 6 h before exposure to 6-OHDA. After incubation for an additional 2 h, Fluo-4 AM was added to each well, and the mixture was incubated at 37 °C for 30 min. Intracellular $Ca^{2+}$ was analyzed by the intensity of fluorescence emitted with $Ca^{2+}$-conjugated Fluo-4 AM (with excitation at 485 nm and emission at 520 nm) using a microplate reader (Bio-Tek Instruments), and concentrations are reported as multiples of the control [29].

### 2.12. Activity analysis of caspases 3 and 9

mNGF-Differentiated PC12 cells were treated with the test compounds for 6 h before exposure to 6-OHDA. After incubation for an additional 6 (caspase 3) or 4 h (caspase 9), activities of caspases 3 and 9 were measured with fluorometric assay kits, according to the manufacturer's instructions for caspase 3 (with excitation at 360 nm and emission at 440 nm) (Sigma, St. Louis, MO, USA) and for caspase 9 (with excitation at 400 nm and emission at 505 nm) (Biovision, San Diego, CA, USA), to detect the fluorescence emitted from the hydrolysis of the caspase 3 or 9 substrate.

### 2.13. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). T-test was performed for animal study and multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test for the cell study. Statistical analyses were performed with GraphPad Prism 5.0 software (San Diego, CA, USA).

## 3. Results

### 3.1. Malondialdehyde (MDA) contents in plasma and brain extracts

High levels of polyunsaturated fatty acids are found in brain cell membranes and react as substrates for lipid peroxidation [30]. Levels of MDA, one of the final products of the peroxidation caused by oxidative stress, were assessed in the plasma and brain extracts of different groups. Animal weights among groups did not significantly differ during the experiments (data not shown); however, plasma MDA contents of the groups changed over the experimental period (Fig. 1A). Compared to the D-galactose group (the control), the blank group showed significantly lower MDA contents ($p < 0.05$) at the 4- and 8-week time intervals. After the 8-week intervention with a concurrent D-galactose injection, the aqueous extract (UHH) and 95% ethanol extract (UHE) interventions showed significantly lower MDA contents ($p < 0.05$) compared to the D-galactose group as shown in Fig. 1A. Thus, the UHH or UHE intervention for 8 weeks may reduce the increased plasma MDA contents due to the 8-week D-galactose subcutaneous injection. Therefore, levels of MDA (Fig. 1B) were determined in tissue extracts of the brain after sacrifice. The blank group showed the lowest MDA content in brain extracts among all groups. It was found that interventions with 80 mg/kg UHH or UHE decreased MDA contents in brain extracts; however, only UHE showed a significant ($p < 0.05$) difference from the control group (Fig. 1B). Thus, the UHH or UHE intervention could decrease MDA contents in the plasma or in brain tissues of model mice that had undergone long-term D-galactose induction.

### 3.2. Learning behaviour evaluations

In the last week of the intervention, mice in each group were evaluated by step-through passive avoidance tests for short-term learning and memory capacities (Fig. 1C). On the training day (day 1), it was found that mice stepped quickly toward the dark chamber and showed no significant difference in latency among the groups ($p > 0.05$). After having received an electric foot shock in the dark chamber previously, the mice in the blank group on day 3 remained in the illuminated chamber for the maximal 300-s latency time; however, mice in the control group stepped toward the dark chamber and showed a significantly different latency (85.7 ± 29 s) compared to that of the blank ($p < 0.05$). It was found that the 80 mg/kg UHH and 80 mg/kg UHE interventions could respectively increase the latency to 152.1 ± 38.6 and 215.1 ± 51.9 s; however, only UHE showed a significant ($p < 0.05$) difference from the control group (Fig. 1C). Thus, the UHE intervention improved the short-term learning and memory capacities of model mice that had undergone long-term D-galactose induction. The present results of learning behavior were positively correlated with the MDA contents (an oxidative stress marker) in brain extracts as shown in Fig. 1C.

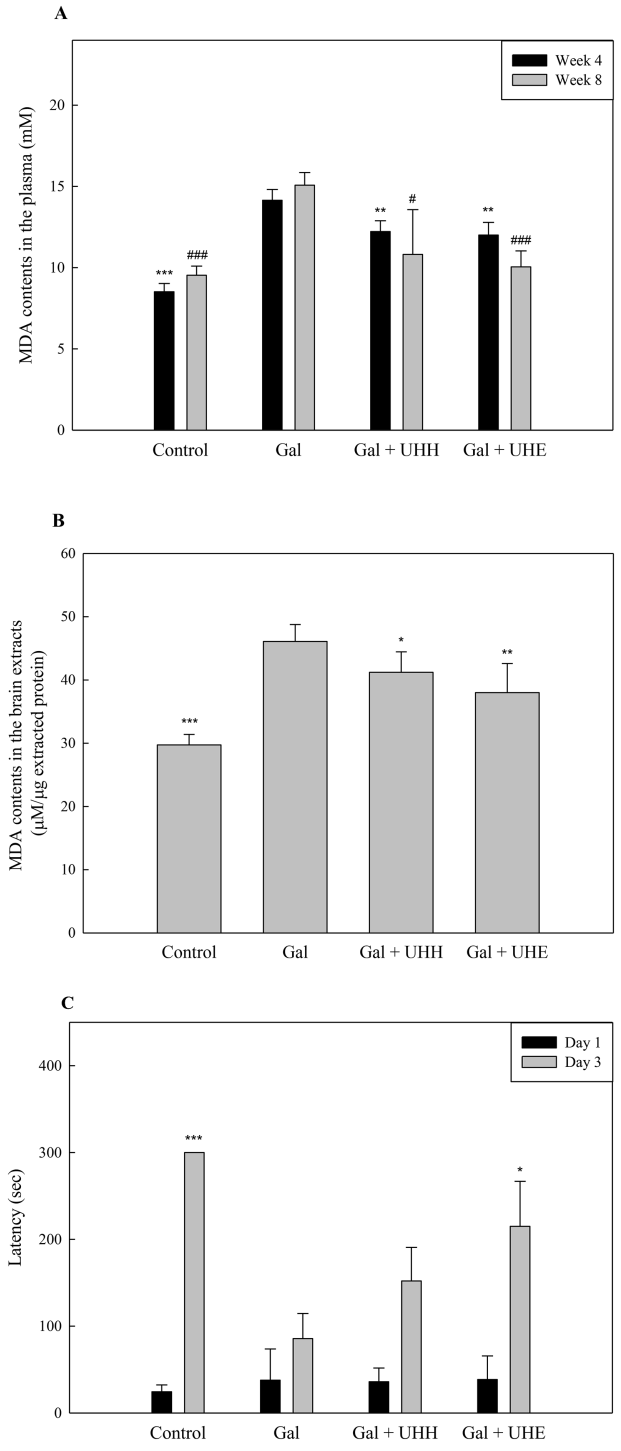



**Fig. 1 – Effects of *U. hirsuta* extracts on MDA content and learning behavior in male BALB/c mice. The animals were divided into groups: D-galactose-induced mice (Gal) with 95% ethanol (UHE) or water (UHH) interventions for 8 weeks. MDA content was evaluated from (A) the plasma of mice at 4-week intervals and (B) brain extracts after the mice were euthanized. (C) Learning behavior was evaluated using a step-through passive avoidance test, and the parameters of latency (time in seconds that a mouse stayed in the illuminated chamber on days 1 and 3) were recorded for comparison with the controls. Data are expressed as the mean ± SD. *, $^{\#}p < 0.05$, **, $^{\#\#}p < 0.01$, and ***, $^{\#\#\#}p < 0.001$ when compared with the controls at 4 weeks and 8 weeks respectively. UHH, aqueous extract of *U. hirsuta*; UHE, 95% ethanol extract of *U. hirsuta*.**

### 3.3. *Cytotoxicity and neuroprotection of* U. hirsuta *in mNGF-differentiated PC12 cells*

To evaluate the neuroprotective properties of *U. hirsuta*, we needed to be clear about its cytotoxicity in mNGF-differentiated PC12 cells. 100 μg/mL UHE and UHH were individually added to cells and incubated for 6 h. Cell viability was evaluated with the WST-8 assay, and cells with a viability of ≥80% were considered for further experiments (Table 1) Following this, we checked the neuroprotective effect by incubating PC12 cells with the neurotoxin, 6-OHDA (300 μM), for 24 h first and then with same extracts at the same concentrations. We found that UHE (48.1%) had a greater neuroprotective effect (Table 1). We took the crude UHE and separated three different fractions (EtOAc, *n*-BuOH and aqueous fraction) from it and again checked the cytotoxicity and the neuroprotection of those fractions. We found that the cell viability of *n*-BuOH and aqueous fractions was higher than 80% (Fig. 3A), among which the best results were seen with the *n*-BuOH fraction which exhibited the highest neuroprotective effect (53.7%) as well (Table 1). We thus further used this for compound isolation.

### 3.4. *Identification of major compounds in the n-BuOH fraction from* U. hirsuta

According to cell viability and neuroprotective outcomes in 6-OHDA-induced mNGF-differentiated PC12 cells, the *n*-BuOH fraction of *U. hirsuta* was found to be highly effective. Diaion HP-20, C-18, MCI CHP-20P column chromatography and C-18 semi-preparative HPLC were used to isolate and purify compounds from the active *n*-BuOH fraction. Ultimately three major compounds based on physical and spectroscopic characteristics (suppl. data 1) were identified as 5$\beta$-carboxystrictosidine (**1**) [31], chlorogenic acid (**2**) [32], and uncarine A (**3**) [33] (Fig. 2), and these were further used to check the neuroprotectivity.

### 3.5. *Cytotoxicity and neuroprotection of isolated compounds from* U. hirsuta *in mNGF-differentiated PC12 cells*

The cytotoxicity and neuroprotectivity of compounds isolated from the *n*-BuOH fraction, including 5$\beta$-carboxystrictosidine (**1**), chlorogenic acid (**2**), and uncarine A (**3**), were evaluated. mNGF-differentiated PC12 cells were individually incubated with **1**, **2**, and **3** (100 μM) for 6 h. Cell viability was determined using the WST-8 assay. The cell viability of cells treated with **1** and **2** was found to be >80%, so we considered these two to be neuroprotective compounds (Table 2). Cells pre-treated with the above two compounds were then incubated with 6-OHDA (300 μM) for 24 h, and we found that the neuroprotective effects of **1** and **2** were 78.1% and 86.9%, respectively (Table 2). These results demonstrated that **1** and **2** are neurocytoprotective compounds. We next observed the correlation

**Table 1 – Cytotoxic and neurocytoprotective effects of the extracts from *U. hirsuta* in mNGF-differentiated PC12 cells.**

| Sample | Cell viability (%) | |
|---|---|---|
| | Sample only | 6-OHDA treatment |
| Control | 100.0 ± 1.5** | 50.3 ± 4.2## |
| UHH | 100.0 ± 2.9 | 55.5 ± 2.3* |
| UHE | 101.1 ± 2.8 | 74.2 ± 0.9** |
| EtOAc | 69.0 ± 1.5## | – |
| *n*-BuOH | 92.5 ± 5.9# | 77.0 ± 0.5** |
| Aqueous | 85.4 ± 3.1## | 73.4 ± 4.2** |

Data are expressed as a percentage of the control group. #$p < 0.05$ and ##$p < 0.01$ compared to the control (sample only). *$p < 0.05$ and **$p < 0.01$ compared to the control with 6-OHDA treatment. Sample concentration: 100 μg/ml. UHH: aqueous extract of *U. hirsuta*; UHE: 95% ethanol extract of *U. hirsuta*; EtOAc: ethyl acetate fraction from UHE; *n*-BuOH: *n*-butanol fraction from UHE; aqueous: aqueous fraction from UHE.

**Table 2 – Cytotoxic and neurocytoprotective effects of isolated compounds from *U. hirsuta* in mNGF-differentiated PC12 cells.**

| Sample | Cell viability (%) | | $IC_{50}$ (μM) |
|---|---|---|---|
| | Sample only | 6-OHDA treatment | |
| Control | 100.0 ± 5.5** | 53.2 ± 3.5## | |
| Compound (**1**) | 102.6 ± 7.4# | 89.7 ± 1.7** | 55.2 |
| Compound (**2**) | 92.6 ± 2.5## | 97.1 ± 0.4** | 39.7 |
| Compound (**3**) | 79.0 ± 3.7## | – | |

Data are expressed as a percentage of the control group. #$p < 0.05$ and ##$p < 0.01$ compared to the control. **$p < 0.01$ compared to the 6-OHDA group. Sample concentrations: 100 μM. Compound (**1**): 5β-carboxystrictosidine; compound (**2**): chlorogenic acid; compound (**3**): uncarine A.

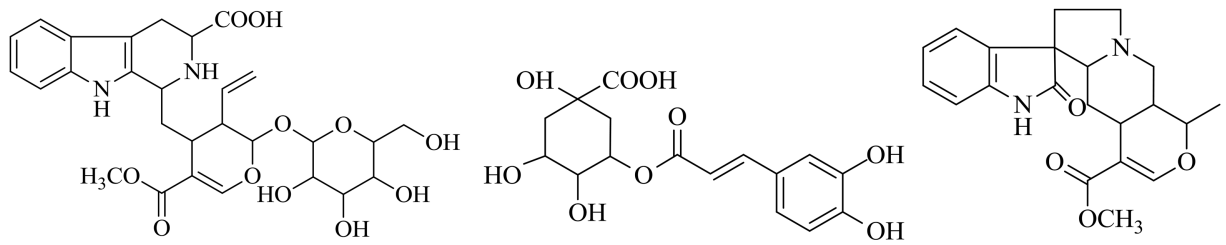
between concentrations of **1** and **2** and the neuroprotectivity of mNGF-differentiated PC12 cells. mNGF-differentiated PC12 cells were incubated with varying concentrations (12.5, 25, 50, and 100 μM) of **1** and **2**, respectively, for 6 h and then incubated with 6-OHDA (300 μM) for an additional 24 h. We observed that the neurocytoprotective effect of both compounds increased in dose-dependent manners, implying that the neurocytoprotective effects of **1** and **2** were directly proportional to the concentration, and the $IC_{50}$ for **1** was 55.2 μM and that for **2** was 39.7 μM (Table 2).

### 3.6. *Antiapoptotic effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on mNGF-differentiated PC12 cells*

Apoptosis plays a key role in the progression of several neurologic disorders, such as AD, PD, Huntington's disease, and amyotrophic lateral sclerosis, as demonstrated by various studies on animal models and cell lines [7]. In the present study, we aimed to evaluate whether the active compounds were capable of combating 6-OHDA-induced apoptosis. In order to check the neuroprotective effects of **1** and **2**, apoptosis was induced in mNGF-differentiated PC12 cells by 6-OHDA. mNGF-differentiated PC12 cells were incubated with different concentrations (12.5, 25, 50, and 100 μM) of these two compounds for 6 h, after which 6-OHDA was added and incubated for 24 h. We detected the percentage of apoptosis by flow cytometry using Annexin V-FITC and PI double-staining. Quadrant sections Q1–Q4 were respectively representative of necrosis, late apoptosis, survival, and early apoptosis of cells (Fig. 3A). We considered Q2 and Q4 for the total apoptosis result, and it was seen that apoptosis at each concentration of **2** was less than that of **1**, indicating that the protective effect of **2** was greater than that of **1**. $IC_{50}$ values were 68.10 and 38.10 μM for **1** and **2**, respectively (Fig. 3B). This result indicates that these two compounds interacted with the neurotoxin by inducing an anti-apoptotic effect.

### 3.7. *Effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on the nuclear morphology of mNGF-differentiated PC12 cells*

Apoptosis can be induced through the targeting the different organelles like mitochondria or nuclei leading to the changes such as in nuclear morphology. We observed the effects of the active compounds on 6-OHDA-induced changes in the nuclear morphology of mNGF-differentiated PC12 cells using 4′,6-diamidino-2-phenylindole (DAPI) staining. mNGF-differentiated PC12 cells were incubated with various concentrations (12.5, 25, 50, and 100 μM) of **1** or **2** for 6 h. 6-OHDA (300 μM) was added to cells and incubated for 24 h. Confocal microscopy was used to observe the morphology of cells, and we found that cells with fragmented nuclei were apoptotic cells (Fig. 4). This suggested that **1** and **2** have the ability to protect mNGF-differentiated PC12 cells from 6-OHDA-




5β-Carboxystrictosidine (**1**) Chlorogenic acid (**2**) Uncarine A (**3**)

**Fig. 2 – Structures of the compounds isolated from *U. hirsuta*.**

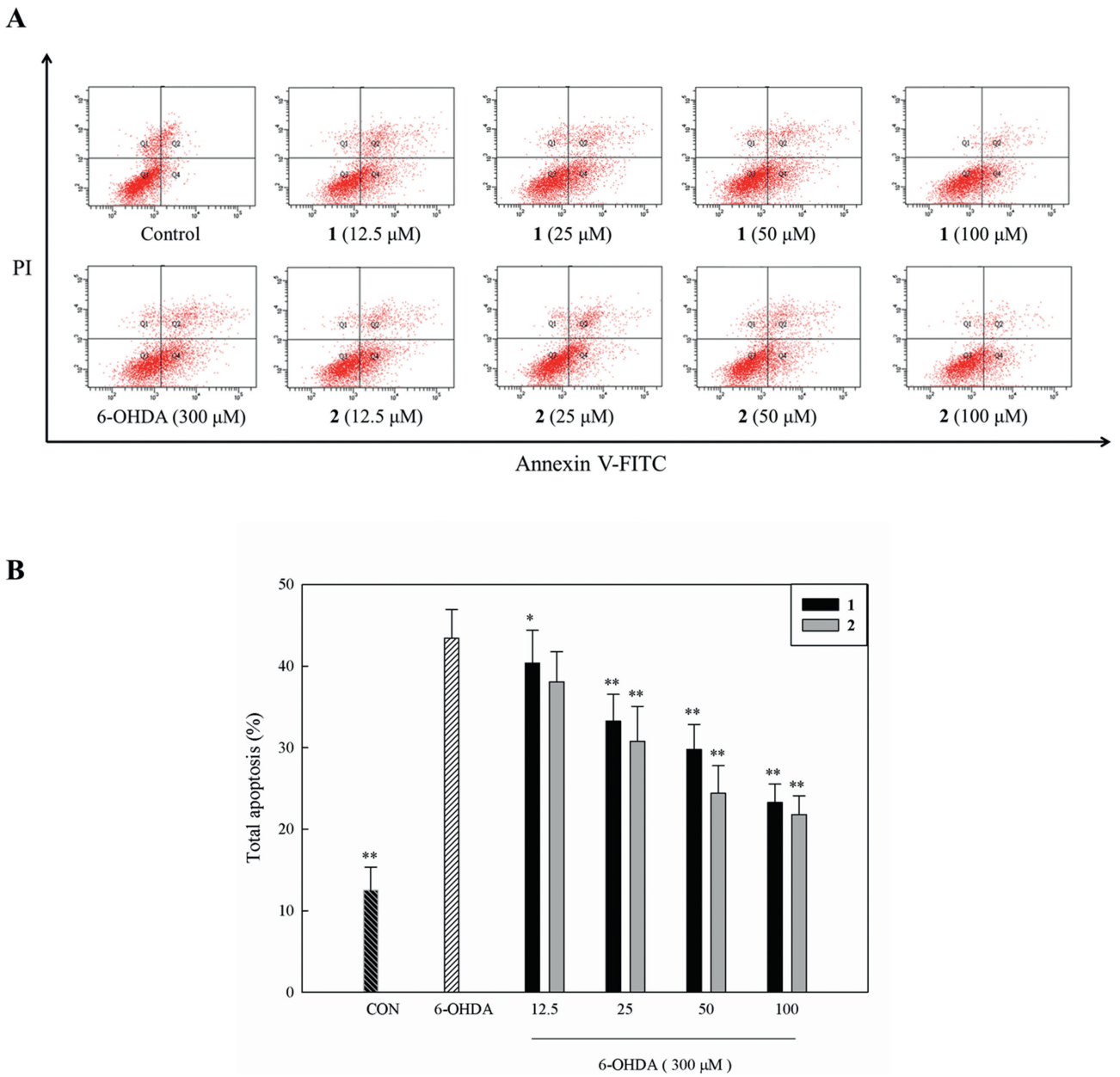



**Fig. 3 – Neuroprotective effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) against 6-OHDA-induced apoptosis in mNGF-differentiated PC12 cells. The cells were incubated with different concentrations (12.5, 25, 50, or 100 μM) of the compounds, and 6-OHDA (300 μM) was added subsequently. (A) The percentage of apoptotic cells was determined through flow cytometry using Annexin V-FITC and propidium iodide (PI) double-staining. Q1 – Q4 respectively represent necrosis, late apoptosis, survival, and early apoptosis cells. (B) Q2 and Q4 were evaluated for the effects of apoptosis. 100% cell death was considered total apoptosis and necrosis. *$p < 0.05$ and **$p < 0.01$ when compared with the 6-OHDA group.**

induced apoptosis and confirms the earlier proven point that these are anti-apoptotic agents.

### 3.8. *Effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on intracellular ROS in mNGF-differentiated PC12 cells*

Oxygen is imperative for life, and imbalanced metabolism and excess ROS generation result in a range of disorders. The toxicity of free radicals contributes to protein and DNA injuries, inflammation, tissue damage, and subsequent cellular apoptosis. ROS are a very dangerous threat as they attack at the cellular level but have effects far beyond that level ranging up to the organ level. One of the modes of actions of the neurotoxin, 6-OHDA, inducing cytotoxicity is by the production and accumulation of ROS [34]. A set of experiments was designed to check the effects of **1** and **2** on ROS accumulation in mNGF-differentiated PC12 cells. mNGF-Differentiated PC12 cells were incubated with different concentrations (12.5, 25, 50, and 100 μM) of **1** and **2**

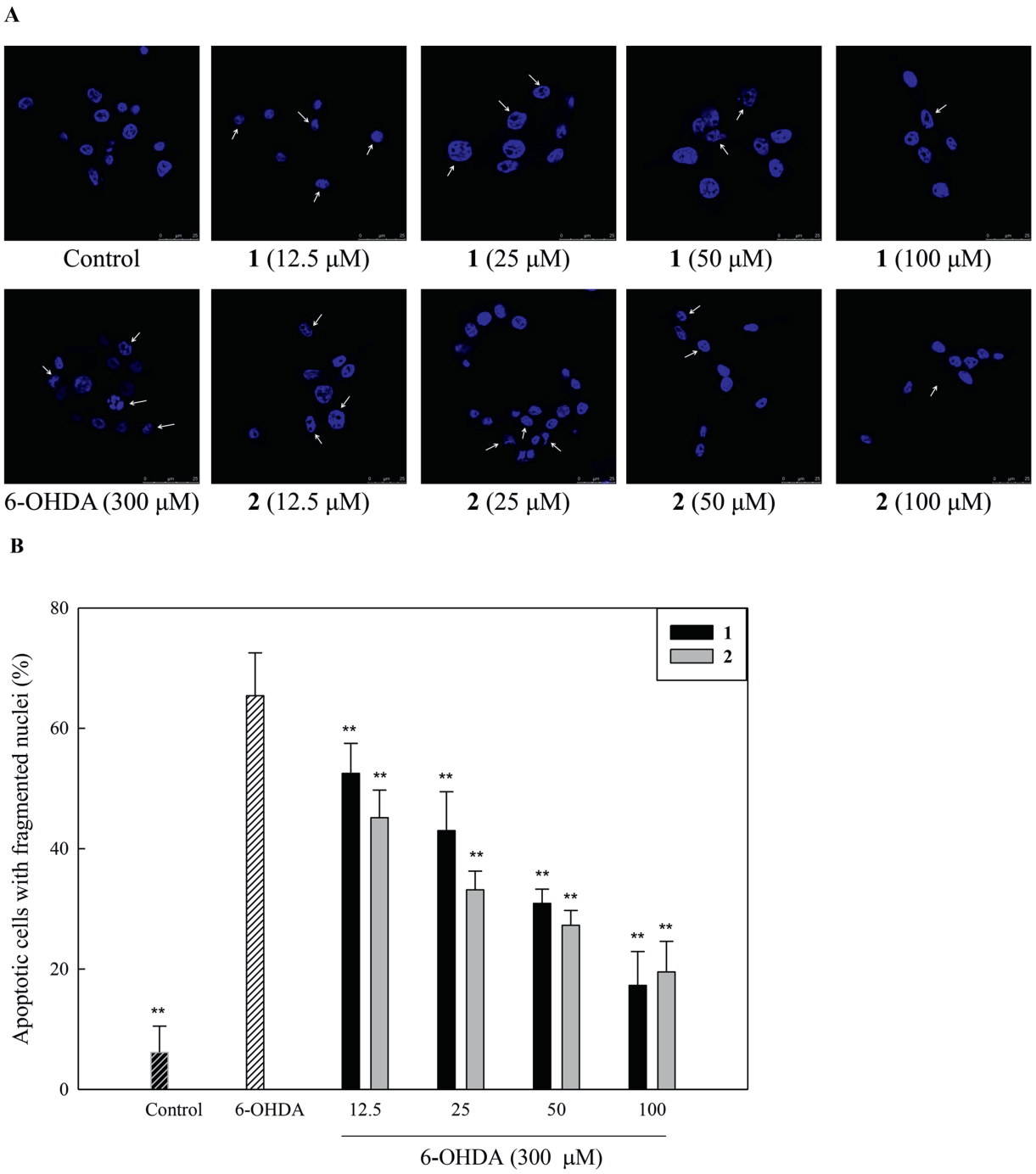



**Fig. 4 – Effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on 6-OHDA-induced changes in the nuclear morphology of mNGF-differentiated PC12 cells. The cells were incubated with different concentrations (12.5, 25, 50, or 100 μM) of the compounds, followed by the addition of 6-OHDA (300 μM). (A) The nuclear morphology was analyzed using confocal microscopy. White arrows indicate apoptotic cells, which were visualized using Image J. Scale bars, 25 μm. (B) Quantitative elucidation of the apoptotic cells.**

individually for 6 h. Cells were incubated with 6-OHDA (300 μM) for 24 h. Intracellular ROS levels were determined by measuring the fluorescence with excitation at 485 nm and emission at 535 nm. The intracellular ROS level increased 1.57-fold after the addition of 6-OHDA compared to the control. It was observed that both **1** and **2** were able to decrease intracellular ROS levels in dose-dependent manners. $IC_{50}$ values for **1** and **2** were 24.5 and 19.7 μM, respectively (Table 3).

### *3.9. Effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on 6-OHDA-induced intracellular calcium concentrations ($Ca^{2+}$) in mNGF-differentiated PC12 cells*

As the antiapoptotic and antioxidant properties of **1** and **2** were confirmed, it seemed necessary to observe other aspects of interactions or mechanisms of occurrence of apoptosis and

**Table 3 – Effect of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on 6-OHDA-induced accumulation of intracellular ROS, intracellular $Ca^{2+}$ levels, caspases 3 and 9 activities in mNGF-differentiated PC12 cells.**

| Sample | Intracellular ROS level (fold) | Intracellular $Ca^{2+}$ level (fold) | Caspase 3 activity (fold) | Caspase 9 activity (fold) |
|---|---|---|---|---|
| Control | 1.00 ± 0.01** | 1.00 ± 0.10** | 1.00 ± 0.03** | 1.00 ± 0.04** |
| 6-OHDA (300 μM) | 1.57 ± 0.03 | 1.80 ± 0.04 | 2.61 ± 0.03 | 1.85 ± 0.02 |
| Compound (1) | | | | |
| 12.5 μM | 1.43 ± 0.10 * | 1.58 ± 0.05** | 2.23 ± 0.09** | 1.57 ± 0.04** |
| 25 μM | 1.22 ± 0.06** | 1.47 ± 0.06** | 1.62 ± 0.06** | 1.39 ± 0.06** |
| 50 μM | 1.10 ± 0.01** | 1.38 ± 0.01 | 1.33 ± 0.03** | 1.17 ± 0.03** |
| 100 μM | 1.03 ± 0.05** | 1.17 ± 0.09** | 1.06 ± 0.04** | 1.07 ± 0.04** |
| Compound (2) | | | | |
| 12.5 μM | 1.41 ± 0.03** | 1.51 ± 0.06** | 2.06 ± 0.04** | 1.51 ± 0.06** |
| 25 μM | 1.13 ± 0.01** | 1.45 ± 0.01** | 1.65 ± 0.06** | 1.27 ± 0.03** |
| 50 μM | 1.11 ± 0.02** | 1.16 ± 0.04** | 1.34 ± 0.05** | 1.13 ± 0.05** |
| 100 μM | 1.08 ± 0.02** | 1.08 ± 0.04** | 1.15 ± 0.06** | 1.03 ± 0.03** |

Data are expressed as multiples of the increase in the fluorescence intensity ($n = 3$). *$p < 0.05$, **$p < 0.01$ compared to the 6-OHDA group. Compound (**1**): 5β-carboxystrictosidine; compound (**2**): chlorogenic acid.

determine the effects of **1** and **2** on the mode of induction, as 6-OHDA has the capacity to induce an elevation of intracellular $Ca^{2+}$, which further induces PC12 cell apoptosis [35]. So, the effects of **1** and **2** on intracellular $Ca^{2+}$ were analyzed by measuring the fluorescence intensity emitted by Fluo-4/$Ca^{2+}$ conjugates. mNGF-Differentiated PC12 cells were incubated with **1** and **2** individually at different concentrations (12.5, 25, 50, and 100 μM) for 6 h. After this, 6-OHDA (300 μM) was added and incubated for 2 h. A 1.8-fold increase in the fluorescence intensity was observed with the addition of 6-OHDA, compared to the control. Pre-treatment of PC12 cells with **1** or **2** produced a decrease in the fluorescence intensity in dose-dependent manners. $IC_{50}$ values for **1** and **2** were calculated to be 46.9 and 27 μM, respectively (Table 3), showing that both compounds were effective in reducing intracellular $Ca^{2+}$ levels induced by 6-OHDA. Hence one more characteristic activity was confirmed.

### 3.10. *Effects of 5β-carboxystrictosidine (1) and chlorogenic acid (2) on the activities of caspases 3 and 9 in mNGF-differentiated PC12 cells*

Information about apoptosis and its mode of action was obtained using the preceding experiments without answering the questions about the effects of **1** and **2** on the molecules involved in the process of apoptosis and their activities. Caspases function as central regulators of apoptosis, and caspase 3 is a very important regulator of apoptosis in neural cells. Caspase 3 is activated in apoptotic cells by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways, and active caspase 9 is required to recruit caspase 3 [36]. The genetic deletion of caspases 3 and 9 prevented apoptosis after mNGF deprivation and importantly allowed these neurons to recover and survive long-term following re-addition of mNGF, and it was found that 6-OHDA induced caspase 3 activation. To check the effects of **1** and **2** on the activities of caspases 3 and 9, mNGF-differentiated PC12 cells were incubated for 6 h with different concentrations of **1** or **2** (12.5, 25, 50, and 100 μM). After this, 6-OHDA at 300 μM was added for 6 h (for caspase 3) and 4 h (for caspase 9). Activities of caspases 3 and 9 were determined by measuring the fluorescence with excitation at 360 nm and emission at 440 nm (for caspase 3) and excitation at 400 nm and emission at 505 nm (for caspase 9). It was seen that 6-OHDA was able to increase the activities of caspases 3 and 9. $IC_{50}$ values of **1** for caspases 3 and 9 were 25.6 and 24.5 μM, and those for **2** with regard to caspases 3 and 9 were 19.4 and 16.3 μM, respectively (Table 3).

## 4. Discussion

In this study, we evaluated the neuroprotective activity of *U. hirsuta* on the experimental models designed for neurodegeneration based on the administration of neurotoxins. We attempted to identify the mode of action of neuroprotection offered by isolated active compounds **1** and **2** from *U. hirsuta*. Chronic exposure to D-galactose induces stress and causes an increase in MDA and a decrease in total antioxidative capabilities, and total superoxide dismutase and glutathione peroxidase activities [6]. Elevated levels of MDA are the outcome of stress induction [37]. It was found that increased levels of MDA due to oxidative stress can cause DNA damage [38,39]. MDA results from the lipid peroxidation of polyunsaturated fatty acids due to ROS. This highly reactive aldehyde yields advanced lipoxidation end-products [40]. High levels of MDA were found in non-surviving patients with brain injury compared to surviving ones [41]. *U. hirsuta* extract has the potential to reduce the MDA content of plasma and brain tissues in D-galactose-induced mice. Determining whether the active constituent is able to cross the blood–brain barrier (BBB) is critical. Numerous studies have been conducted to explore the pharmacokinetics of chlorogenic acid, one of the active compounds in our study. A study on Charles–Foster rats revealed that the concentration of chlorogenic acid was four times higher in the brain when administered intranasally rather than intravenously [42]. The neuroprotective effects of *U. hirsuta* are contributed by 5β-carboxystrictosidine (**1**) and chlorogenic acid (**2**), and as reported previously, compound **2** can cross the BBB [42].

In the current *in vitro* study model, we analyzed the effect of isolated compounds on oxidative stress. The compounds we isolated from *U. hirsuta,* for **1**, we were unable to find any references for its bioactivities. It appears that **2** exhibits its neuroprotective effect by scavenging free radicals, inducing the endogenous antioxidant system, inhibiting apoptotic processes, and activating protein kinases. Also in our study, **2** showed antiapoptotic activity in mNGF-differentiated PC12 cells. Apoptosis can be triggered by many different stimuli like oxidative stress, toxins, insufficient growth factors, and an influx of calcium through plasma membrane channels or release from the endoplasmic reticulum. All factors responsible for apoptosis are directly or indirectly interconnected; for example, the release of calcium from the endoplasmic reticulum leads to the release of cytochrome *c* from mitochondria of cells, which further activates caspases and nucleases leading to the execution of apoptosis [43–45]. In our study, we observed that both **1** and **2** lead to the drastic decrease in the levels of intracellular $Ca^{2+}$, thus leading to inhibition of the release of cytochrome *c*, resulting in the inhibition of apoptosis.

Oxidative stress is one more factor related to apoptosis; usually ROS and all of the superoxides are dealt with by antioxidants like glutathione, carotenoids, and ascorbic acid, but when these dangerous molecules exceed the control of antioxidants, this leads to a condition of oxidative stress in cells, resulting in DNA damage and destruction of lipids and proteins, and ends in cell death via apoptosis [46,47]. Mitochondria play a vital role in inducing apoptosis through oligomerization of adapter proteins and procaspases which further result in auto-activation of initiator caspases; all this begins with the apoptotic activation of mitochondria [48]. This is how intrinsic oxidative stress is usually connected to age through diminished antioxidant activity and mitochondrial dysfunction. Several markers of oxidative stress were found in postmortem examinations of brains from patients suffering with neurodegenerative disorders [49]. Brains of PD patients showed evidence of oxidative stress, like protein oxidation, lipid peroxidation [50]. So we also studied the effects of **1** and **2** on intracellular ROS, and both compounds were able to scavenge ROS and act as antioxidants.

In mammalian cells, apoptosis is regulated by the B-cell lymphoma (Bcl)-2 family of proteins, the adaptor protein apoptotic protease activating factor (APAF)-1, and the cysteine protease caspase family [51]. This regulation and mechanism of apoptosis are shared by all cells including neurons, although the type of neurons and different developmental stages express different combinations of Bcl-2 and caspases, which are regulatory specific. We analyzed the effect of **1** and **2** on caspases, and as mentioned earlier, cytochrome *c* activates caspases. More specifically, it activates caspase 9, which further activates caspase 3 and leads to apoptosis. On observing the effects of **1** and **2** on the activities of caspase 3 and 9, we found very promising results supporting the inhibition of caspase activity by **1** and **2**. All results summed up towards single end-point which is apoptosis, but the modes of activation differed, and both compounds were strongly capable of preventing the occurrence of apoptosis.

## 5. Conclusion

Compounds **1** and **2** possess neuroprotective potential, which has several mode of actions. The actual detailed mechanism of interaction is not known to us, but what we observed in our study was that the 95% ethanol extract from *U. hirsuta* has the capability to reverse memory loss, which can serve as a very strong bridge for further studies of the plant extract and its active compounds, as they exhibited promising results in terms of neuroprotection. These activities were reported for the first time making it a very significant study and also in our study the compound **1** was the novel compound isolated with a very potential activity.

## Author contributions

C.M.L., Y.T.L., T.L.L., Z.I, W.C.H., and M.H.L. participated in the research and drafted the manuscript. All authors read and approved the final manuscript.

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## Declaration of Competing Interest

The authors declare no competing financial interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.10.004.

## Abbreviation

| | |
|---|---|
| AD | Alzheimer's disease |
| DAPI | 4′,6-diamidino-2-phenylindole |
| MDA | malondialdehyde |
| mNGF | mouse nerve growth factor |
| 6-OHDA | 6-hydroxydopamine |
| PD | Parkinson's disease |
| PI | propidium iodide |
| ROS | reactive oxygen species |

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