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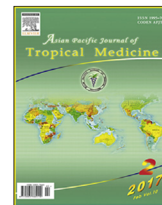

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Neuroprotective activity of two active chemical constituents from *Tinospora hainanensis*

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ABSTRACT

Objective: To determine the chemical structure of the new compound and investigate the protective effects of Tinosporaic acid A and B towards *in-vitro* neuro.

Methods: The structures of two new compounds were established by analyzing its 1D and 2D NMR spectra as well as HRESIMS. Their neuroprotective effects with respect to the antioxidant properties were evaluated by radical scavenging tests and hydrogen peroxide-injured oxidative stress model in PC12 cell lines. Cell morphology of treated PC12 cells was observed by phase contrast microscopy. *In-vitro* MTT assay, lactate dehydrogenase activity assay and oxidative stress markers (intracellular ROS production, MDA level, and caspase-3 activity) were used to evaluate the protective effects against hydrogen peroxide induced cytotoxicity in PC12 cells.

Results: The two new compounds, named Tinosporaic acid A and B, were isolated and identified from the stem bark of *Tinospora hainanensis*. Cell viability studies identified a representative concentration for each extract that was subsequently used to measure oxidative stress markers. Both extracts were able to reverse the oxidative damage caused by hydrogen peroxide, thus promoting PC12 cells survival. The concentration of Tinosporaic acid A and B were 86.34 μg/mL and 22.06 μg/mL respectively, which is neuroprotective for EC50. The results indicated that both of them significantly attenuated hydrogen peroxide-induced neurotoxicity.

Conclusion: The two new compounds isolated from ethanol extracts of *Tinospora hainanensis* are the promising natural ones with neuroprotective activity and needed for further research.

1. Introduction

Tinospora hainanensis (T. hainanensisis) a member of the genus *Tinospora* in the Menispermaceae family, which is widely distributed in sub-tropical and tropical regions of the world [1–3]. This plant is endemic to Hainan Province in China. It is commonly used as a traditional Chinese folk medicine as remedy for joint pain and physical injury [4,5]. In previous studies, chemical investigations have led to the isolation of alkaloids and sterones [6,7]. As an ongoing research, two new sterones, named Tinosporaic acid A and B, together with two known alkaloids were isolated from the stem bark of *T. hainanensis*.

Oxidative stress, defined as a redox imbalance between reactive oxygen species (ROS) along with the free radical cleansing protection procedure, results in the damage of cellular

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contents, including proteins, lipids and DNA [8]. It plays a pivotal role in neuronal damage, and is responsible for the development of several neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [9]. The antioxidant potential of nervous system cells can be exploited as a therapeutic manner for slowing and preventing neurodegeneration. Several intracellular mechanisms help counteract oxidative stress; for example, antioxidant compounds that upregulate the Akt/MAPK/Nrf2 pathway promote the induction of cytoprotective genes, such as detoxifying antioxidant phase-II enzymes [10,11]. Through the model of the neuronal cell death *in vitro* and neuro degeneration in vivo, various natural products have been demonstrated to be neuroprotective.

Our knowledge of the pharmacological properties of *T. hainanensis* is still poor in comparison with that of other natural products. In this paper, we reported the structural elucidation of the new isolates as well as their neuroprotective effects against hydrogen peroxide-induced neurotoxicity *in vitro*. In previous investigations, some *Tinospora* species have been demonstrated to have antioxidant properties arising from their phenolic content [12]. For instance, antioxidant activities of several depsides and depsidones isolated from various *Tinospora* species and *in-vitro* properties of some crude *Tinospora* extracts have been described [13]. Nevertheless, there are few studies of intracellular ROS modulation by *Tinospora* extracts and metabolites, and none has been focused on their protective role in the research on the nervous system-like cells under oxidative stress conditions.

In view of this, our research attempts to identify and isolate *T. hainanensis* with potential antioxidant activities and what's more, to protect against oxidative stress in models of nervous system-like cell lines. The present work focuses on the possible neuroprotective properties of the ethanol extracts from two new compounds of the bark of *T. hainanensis*.

2. Materials and methods

2.1. Materials

PC12 cells (ATCC) were obtained from Shanghai Institutes for Biological Sciences, CAS (Shanghai, China). DMEM medium, fetal bovine serum and horse serum were provided from Gibco BRL (Grand Island, NY, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), Hydrogenperoxide (H_2O_2) solution (30%w/w), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylicacid (trolox), 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and all other reagents were obtained from Sigma–Aldrich (St Louis, MO, USA). Caspase-3 apoptosis kit was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Lactate dehydrogenase (LDH) assay kit and Lipid Peroxidation (MDA) assay kit were obtained from Jiancheng Institute of Biological Engineering (Nanjing, China).

2.2. Instrumental detections

UV spectra were recorded on a Shimadzu UV2550 spectrometer. IR spectra were measured on a FTIR-8400S spectrometer. One-dimensional (^{1}H, 13C-APT) and two-dimensional (^{1}H–^{1}H COSY, HSQC, HMBC) NMR experiments were performed on Bruker AV Ⅲ 600 spectrometers operating at 600 MHz for 1H and 150 MHz for 13C, respectively (TMS an internal standard). Chemical shifts are expressed in δ (ppm) referenced to solvent peaks at δH 7.26 and δC 77.2 for CDCl3, and coupling constants are in Hz. HRESIMS spectra were obtained from a Thermo Scientific LTQ-Obitrap XL instrument (Thermo Scientific, Bremen, Germany). Silica gel columns (200–300 mesh, Qingdao Marine Chemistry Co. Ltd) were used for separations. HPLC chromatography was performed on Agilent 1100 with a column of YMC-pack ODS-A [(250 × 10) mm, 10 μm]. Mixtures of MeOH/H_2O were used as the eluents. All solvents used were of analytical grade (Beijing Chemical Works, Beijing, P. R. China).

2.3. Plant material

The stem bark (4.0 kg) of *T. hainanensis* was collected in July 2013 from Haikou city, Hainan Province, and was identified by Prof. Naikai Zeng, School of Pharmaceutical Science, Hainan Medical University, Haikou City, Hainan Province, P. R. China. A voucher specimen (No. T2103-1) was deposited in the Herbarium of Institute of Hainan Medical University.

2.4. Extraction and isolation

The air-dried stem bark of *T. hainanensis* (5.0 kg) was extracted with 70% EtOH (3 × 10.0) L under reflux for (1.5 × 3) h. The ethanol extract was filtered and concentrated under reduced pressure to yield a crude extract (500.0 g), which was suspended in distilled H_2O (5.0 L) and then successively partitioned with hexane (3 × 2.0) L and ethyl acetate (3 × 2.0) L to afford EtOAc-soluble fraction (150.0 g). The EtOAc-soluble fraction was subjected to silica gel chromatography [1 000 g, 200–300 mesh, (10 × 110) cm] eluting with a gradient of EtOAc in hexane (0:1, 20:1, 10:1, 5:1, 2:1, v/v) to give five fractions (Fr. A–E) monitored by TLC. Compound 1 (10.0 mg) and 2 (8.0 mg) was obtained from Fr. B (5.5 g) by a silica gel chromatography [100 g, 200–300 mesh, (5 × 20) cm] eluted with a mixture of hexane/acetone (85:15, v/v), followed by preparative HPLC eluting with MeOH–H_2O (68:32).

2.5. Free radical scavenging activities

Oxygen radical antioxidant capacity (ORAC) assay was implemented as previously described [14]. Dilutions of samples and Trolox (reference control, water-soluble vitamin E) were incubated in opaque 96-well plates at 37 °C for 10 min. Following this period of time, AAPH was put into to the mixture. Fluorescence was measured by Luminescence Microplate Readers (Thermo scientific, USA) with λexc 485 nm and λem 520 nm. The area under the curve (AUC) was calculated for each sample and in comparison with that of Trolox. ORAC values are expressed as μmol Trolox equivalent (TE)/mg sample. DPPH analysis was executed following the previously described DPPH method with little modifications [15]. In brief, different concentrations of the extracts were placed in a 96-well plate and a DPPH solution (50 μM) was added to make up a volume of 225 μL/well. The resulting solutions were incubated in darkness for 30 min and their absorbances were read during 517 nm in a Thermo microplate readers apparatus.

2.6. Cell culture and drug treatment

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone) and 100 U/mL penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C [16]. Cells were pretreated with different concentrations of Tinosporaic acid A and B for 1 h, then incubated with 0.2 mM H_2O_2 for 24 h. In these experiments, H_2O_2 was diluted in DMEM. Control group was also administered to the same amount of DMEM. Tinosporaic acid A and B were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO was less than 0.1% (v/v). All experiments were performed in three times. PC12 cells were seeded in a 24-well culture plate at a density of 2×10^5 cells/well and cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The cellular morphology was observed by using an inverted microscope (Olympus, Japan).

2.7. MTT assay

PC12 cells were seeded in 96-well culture plates at an approximate 2×10^4 cells per well and maintained in a CO_2 incubator at 37 °C overnight. Different concentrations of Tinosporaic acid A and B were added into the medium pretreated for 1 h and followed by adding 0.2 mM H_2O_2 to be incubated for another 24 h. Then, 10 μL of 5 mg/mL MTT was added to the medium and incubated for 4 h at 37 °C. After removal of the culture medium, the insoluble purple formazan crystal was dissolved with 100 μL DMSO to be the violet solution [17]. The absorbance was measured at 490 nm with a microplate reader (Bio-Rad instruments, Inc.). Cell viability was expressed in percentage and the control group was considered 100%.

2.8. LDH release assay

PC12 cells were seeded in a 96-well culture plate at 1×10^4 cells/well and LDH leakage in the conditioned medium, an indicator of cellular injury, was used to detect cellular injury with LDH assay kit [18]. According to the manufacturer's instructions, the reaction was initiated by mixing 40 μL of the conditioned medium with pyruvate, and then reacted with 2,4-dinitrophenylhydrazine. After reaction, the absorbance of the each sample was read at 440 nm. Data were normalized to the LDH activity released from control cells.

2.9. Intracellular ROS production assay

ROS production was evaluated by the DCFH-DA method with some modifications [19]. Briefly, after different treatment, cells were collected and incubated with 10 mM DCFHDA for 30 min at 37 °C, then washed with PBS twice. The relative levels of fluorescence were quantified in fluorospectrophotometer (Thermo, USA) with excitation at 488 nm and emission at 530 nm.

2.10. Measurement of lipid peroxidation

After exposure to 0.2 mM H_2O_2 for 24 h, PC12 cells were washed with ice-cold PBS and lysed by using cell lysis buffer. Cell lysates were centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was collected, and Malondialdehyde (MDA) levels were analyzed by specific assay kits according to the manufacturer's protocol [20]. Total protein concentrations of whole cell lysates were measured according to BCA method, using bovine serum albumin as the standard.

2.11. Caspase-3 activity assay

PC12 cells were seeded in the 12-well plates at 1×10^5 cells/well and the activity of caspase-3 like protease in the lysate was measured using a colorimetric caspase-3 assay kit from Santa Cruz [21]. The caspase-3 activity was expressed by value of OD405.

2.12. Statistical analysis

Data were presented with the mean ± standard deviation (SD). Statistical analysis of the data was evaluated by one-way ANOVA followed by a post hoc LSD test. $P < 0.05$ was considered statistical significance.

3. Results

3.1. Phytochemical analysis of Tinosporaic acid A and B

Tinosporaic acid A (1) was obtained as colorless oil. It possessed a molecular formula $C_{16}H_{20}O_6$ as determined by HR-ESI-MS at *m/z* 307.117 6 $[M–H]^-$ (calcd. 307.118 2 $[M–H]^-$). The IR spectrum showed characteristic absorptions attributing to hydroxyl (3380 cm^{-1}), and carbonyl groups (1 752, 1734 cm^{-1}). The ^1H NMR spectrum of 1 exhibited signals attributable to an aromatic ABX system at 7.08 (^1H, dd, J = 8.0, 1.8 Hz, H-6), 7.03 (^1H, d, J = 1.8 Hz, H-2), 6.91(^1H, d, J = 8.0 Hz, H-5), a typical trisubstituted benzene ring. Two low-field doublets observed at δH 7.60 (^1H, d, J = 16.2 Hz, H-7), 6.28 (^1H, d, J = 16.2 Hz, H-8) are characteristic of α and β protons of a trans-double bond conjugated to a carbonyl group. Additionally, the ^1H NMR spectrum showed the existence of five methylene groups at δH 4.20 (^2H, t, J = 6.6 Hz, H-1′), 2.38 (^2H, t, J = 6.6 Hz, H-5′), 1.74 (^4H, m, H-2′, 4′), 1.47 (^2H, m, H-3′), one methoxyl group at δH 3.92 (^3H, s, 3-OCH3). Its 13C-APT spectrum indicated the existence of two carbonyl groups at δC 178.8 (C-6′) and 167.5 (C-9), one phenyl ring at δC 148.2 (C-4), 147.0 (C-3), 127.2 (C-1), 123.2 (C-6), 114.9 (C-5), 109.7 (C-2), two sp2-hybridized carbon atoms at δC 145.0 (C-7) and 115.7 (C-8), five methylenes at δC 64.4 (C-1′), 33.9 (C-2′), 28.6 (C-3′), 25.7 (C-4′), 24.6 (C-5′) were also observed in the 13C-APT. In accordance with the ^1H NMR data, one methoxyl group at δ_C 56.2 (3-OCH_3) was clearly figured out.

The ^1H–^1H COSY spectrum suggested that all aliphatic methylene protons were a contiguous spin system comprising H-1′, H-2′, H-3′, H-4′ and H-5′ in the molecule (Figure 2). In the HMBC experiment, the correlations from δ_H 7.60 (H-7) to δ_C 167.5 (C-9), 127.2 (C-1), 123.2 (C-6), 115.7 (C-8), and 109.7 (C-2) and correlations from δH 3.92 (3-OCH3) to δ_C 147.0 (C-3) suggested the presence of a trans-feruloyl group. Furthermore, the HMBC correlations from δH 2.38 (H-5′), 1.74 (H-4′) to δ_C 178.8 indicated linkage from C-1′ to C-6′. Additionally, the HMBC correlations from δH 4.20 (H-1′) to δ_C 167.5 (C-9) indicated linkage between C-1′ and C-9. Therefore, the structure of **1** was established with a trivial name of Tinosporaic acid A.

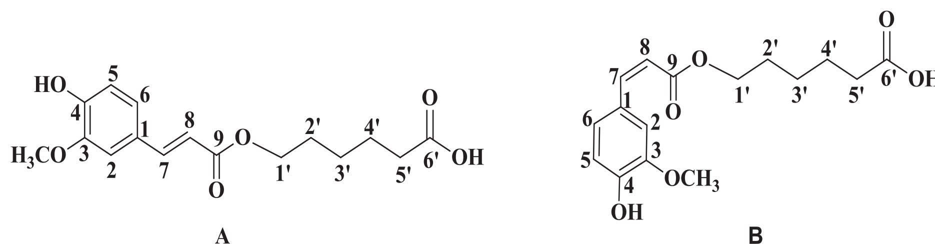

Figure 1. Chemical structures of Tinosporaic acid A and B.

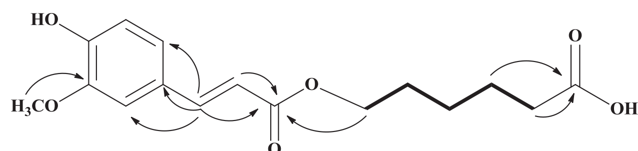

Figure 2. Key HMBC (→) and ^{1}H–^{1}HCOSY (–) correlations of 1.

Tinosporaic acid B (2) was purified as colorless oil. Its molecular formula $C_{16}H_{20}O_6$ was determined by the HR-ESI-MS data, the same as 1. ^{1}H and ^{13}C NMR data were superposable upon those of 1, apart from the configuration of $\Delta^{7(8)}$ double bond. The olefinic geometries in 2 was determined to be cis based on its ^{1}H NMR data at δ_H 6.80 (^{1}H, d, J = 12.6 Hz, H-7), 5.80 (^{1}H, d, J = 12.6 Hz, H-8) and the observed NOESY cross peak between H-7 and H-8. Therefore, the structure of **2** was unequivocally established as shown in Figure 1 with a given name of Tinosporaic acid B.

3.2. Compound content in extraction and radical scavenging activities

Final yields (% w/w) of two new compounds isolated from the ethanol extraction, expressed as μg/mg dry extract, are summarized in Table 1. We observed that the chemiluminescence induced by the peroxyl radical generation, initiated by AAPH in the ORAC assay, decreased following addition of ethanol extract of *T. hainanensis*; ORAC values were 1.74 μmol TE/mg sample for Tinosporaic acid A and 0.56 μmol TE/mg sample for Tinosporaic acid B, indicating the different capacities for scavenging peroxyl radicals. A different pattern of DPPH free radical scavenging activity was seen, whereby Tinosporaic acid B had the highest antiradical activity with the lower IC_{50} (Table 1). The distinct behaviors of the extracts in these assays may be explained by the fundamentally different nature of the methods used. The ORAC and DPPH assays are respectively based on hydrogen. The two new compounds may therefore mediate their radical scavenging activities through different mechanisms.

Table 1

Results obtained for two new compounds isolated from the ethanol extraction and free radical scavenging assays (DPPH and ORAC).

Compounds	Yield (% w/w)	DPPH IC_{50} (μg/mL)	ORAC value (TE/mg)
Tinosporaic acid A	2.39 ± 0.21	144.32 ± 11.73	1.74 ± 0.15
Tinosporaic acid B	5.44 ± 0.39	36.37 ± 5.18	0.56 ± 0.06

3.3. Evaluation of cell viability and protection against H_2O_2-induced toxicity

Seven concentrations of Tinosporaic acid A and B, ranging from 5 to 200 μg/mL and 0.5 μg/mL to 80 μg/mL, were tested to determine the effects of single compound in the MTT assay. Cell viability results obtained for the effects of two contents on PC12 cells are shown in Figure 3A and B respectively, and expressed as the percentage of cell viability. The optical density of untreated control cells was considered 100%. Significant loss of cell viability was observed for Tinosporaic acid A at 100 μg/mL and above, while there was no significant influence for Tinosporaic acid B. At this point, five concentrations for each extract were chosen to assess their capacity to protect against oxidative stress and the cellular toxicity of H_2O_2. H_2O_2 decreased cell viability to approximately 40% of control, but Tinosporaic acid A at 40 μg/mL–80 μg/mL and Tinosporaic acid B at 5 –40 μg/mL significantly reversed that effect and enhanced cell viability (Figure 3C and D). The concentration offering the greatest protection against H_2O_2 was then chosen for each extract (80 μg/mL for Tinosporaic acid A and 20 μg/mL for B) and assayed in subsequent experiments.

3.4. Morphological studies and LDH release assay

The morphology was also studied at these concentrations. Cells treated only with H_2O_2 lost their normal morphology, becoming brighter (less viable and attached to culture dish) and more rounded. Pretreatment with both extracts partially prevented these deleterious effects (see pictures in Figure 4A).

The LDH release assay was used to evaluate the integrity of the cell membrane as another parameter reflecting cell viability. The results complement those of the MTT assay and are expressed as LDH released after treatments (taking total intracellular LDH to be 100%). Control cells released 15% of total intracellular LDH (basal conditions), and cells treated with H_2O_2 alone exhibited a greater release, of up to 40% of total LDH. This elevation was partially attenuated at certain extract concentrations. Results of the LDH release assay confirmed the range of concentrations over which both extracts affect cell viability and protects against H_2O_2 damage (Figure 4B and C). A different effect at several concentrations of Tinosporaic acid A was found when comparing their effects on LDH release and MTT reduction; although at 40 μg/mL did not affect cell viability in the MTT assay, it provoked significant LDH release (Figure 4B). Similarly, for Tinosporaic acid B, concentrations between 10 and 40 μg/mL diminished H_2O_2-induced LDH release (Figure 4C). The different activities in the two experiments reflect the different natures of the methods used, whereby the LDH assay assesses cell membrane integrity while the MTT test evaluates mitochondrial reductase functionality.

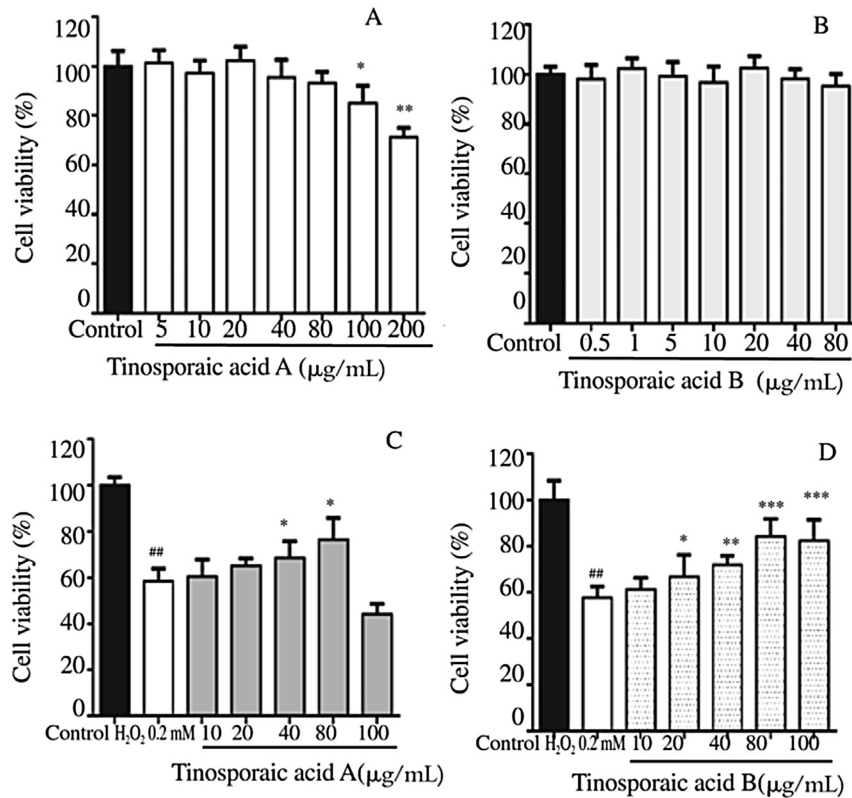

Figure 3. Effects of Tinosporaic acid A and B on PC12 cell viability and protective effects against H_2O_2-induced cell toxicity. (A–B): The direct effect of Tinosporaic acid A and B on PC12 cells viability assessed by MTT assay; (C–D) effect of compound on the viability of damaged PC12 cells elicited by H_2O_2. PC12 cells were incubated with vehicle, different concentrations of Tinosporaic acid A and B for 1 h, and then exposure to 0.2 mM H_2O_2 for 24 h. The data are represented as means ± SD from three experiments. $^{##}P < 0.01$ *vs*. control; $*P < 0.05$, $**P < 0.01$ *vs*. group treated with H_2O_2 alone.

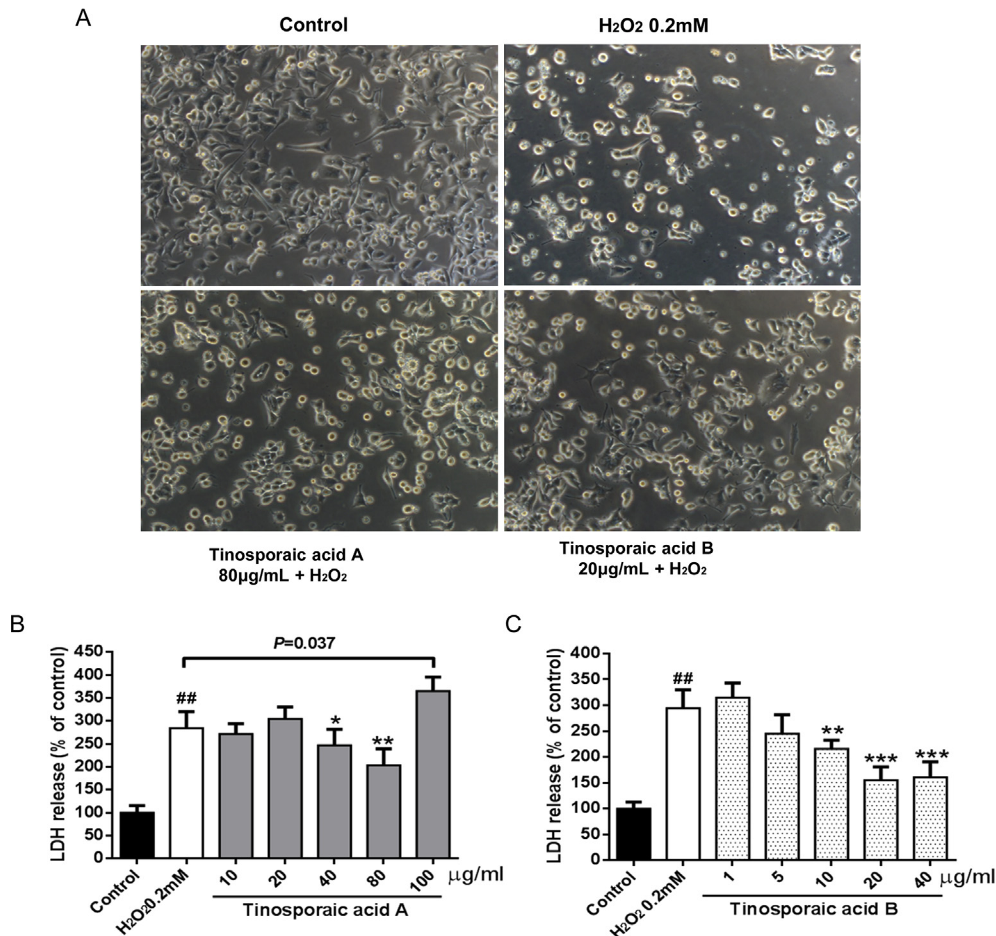

Figure 4. Effect of Tinosporaic acid A and B on morphological changes, and LDH release in PC12 cells injured by H_2O_2. (A) Cell morphology was observed by light microscope (200×); (B–C) Effect of compound on the release of LDH from PC12 cells injured by H2O2. PC12 cells were pretreated by Tinosporaic acid A and B for 1 h and then treated with 0.2 mM H_2O_2 for 24 h. The data are represented as means ± SD from three experiments. $^{##}P < 0.01$ *vs*. control; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. group treated with H_2O_2 alone.

3.5. Intracellular ROS production assay

The effect of exogenous H_2O_2 on intracellular ROS level was assessed by measuring 2,7-dichlorofluorescein fluorescence. PC12 cells exposed to 0.2 mM H_2O_2 presented intracellular ROS levels that markedly increased to approximately 2.25 times in comparison with control cells (100% ROS generation). These results confirm that H_2O_2, under established experimental conditions, induces oxidative stress. Moreover, none of the extracts caused intracellular ROS production when compared with control cells. However, pretreatments with Tinosporaic acid A and B significantly inhibited H_2O_2-induced intracellular ROS generation to (185.42 ± 24.05)% and (121.03 ± 15.16)% (Table 2). These findings may explain the protective role of the two compounds extracts through the reduction of oxidative stress.

Table 2

Effects of Tinosporaic acid A and B on H_2O_2 induced PC12 cytotoxicity markers.

Cell treatment	Intracellular ROS production (%)	MDA levels (nmol/mg protein)	Caspase-3 activity
Control	100.00 ± 21.30	1.35 ± 0.22	100.00 ± 19.04
H_2O_2 0.2 mM	225.36 ± 15.28$^{##}$	3.96 ± 0.47$^{##}$	285.04 ± 35.03$^{##}$
A + H_2O_2	185.42 ± 24.05*	2.94 ± 0.31	224.37 ± 24.52**
B + H_2O_2	121.03 ± 15.16**	1.83 ± 0.25**	142.60 ± 20.75**
Trolox + H_2O_2	147.19 ± 19.33*	2.36 ± 0.14*	187.86 ± 26.43**

The data are represented as means ± SD from three experiments. $^{##}P < 0.01$ *vs*. control; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. group treated with H_2O_2 alone. A = Tinosporaic acid A 80 µg/mL B=Tinosporaic acid B 20 µg/mL.

3.6. Lipid peroxidation

Lipid peroxidation is a major mechanism involving cell insult due to ROS, and also malondialdehyde (MDA) is one of the best known secondary metabolites of lipid peroxidation, becoming widespread as a possible indicator connected with cell injuries [22]. To quantify it, we determined MDA levels by specific kit in the different groups of cells. As illustrated in Table 2, the MDA concentration was significantly higher in H_2O_2-treated PC12 cells than in control cells (3.96 versus 1.35 nmol/mg protein, respectively). Pretreatments with two extracts significantly inhibited the H_2O_2-induced increase of lipid peroxidation in these cells. Tinosporaic acid B was the most active extract and reduced the lipid peroxidation almost to the basal levels found in control cells.

3.7. Determination of caspase-3 activity

Caspase-3 is really a critical enzymatic mediator within external and internal apoptosis pathways. Immediate reduction involving effective caspase-3 contributes to cellular protection from oxidative stress. As soon as it turned out H_2O_2 could induce cellular death through necrosis [23], the possibility that it could possibly likewise promote cell death via apoptosis was examined. We evaluated the effects of two extracts on caspase-3 activity by fluorimetry. As shown in Table 2, exposure of PC12 cells to H_2O_2 produced a remarkable increase of over 285.4% in caspase-3 activity relative to control cells. However, pretreatment with 0.1 mM Trolox (the reference antioxidant) was able to significantly revert this elevation, but not to basal levels. When treated with Tinosporaic acid A and B, cells showed a significant decrease to 224.37% and 142.6%, in caspase-3 activity compared with those exposed to H_2O_2 alone. Consequently, it suggested that the protective effects of *T. hainanensis* extracts are partially mediated by the inhibition of apoptosis.

4. Discussion

Oxidative tension may be generally acknowledged as a factor in numerous ailments. Particularly, central nervous system neurons are generally prone to oxidative stress because of their high rate of oxygen consumption in the brain, the high content of polyunsaturated fatty acids as substrates for lipid peroxidation as well as the presence of iron. H_2O_2 is well known as a cellular toxin. Since H_2O_2 has remarkable membrane permeability, intracellular H_2O_2 can induce detrimental effects on cells. Consequently, we attempted to find neuroprotective compounds with antioxidative activity from natural products using nervous system-like cells (PC12 cells) injured by H_2O_2 as a screening system [24].

For the first time, the neuroprotective activities of ethanol extracts of *T. hainanensis* have been investigated, with respect to their antioxidant actions, in a model of oxidative stress in PC12 cells; such a model was chosen due to the increasingly acknowledged importance of neuron cells in physiological and pathological diseases [25].The chemical structures of these compounds were determined by comparison of their spectroscopic data. Both extracts demonstrated interesting activities in two *in vitro* radical scavenging assays (DPPH and ORAC), suggesting that this will be a possible mechanism accounting for their antioxidant capacity.

We then assessed the antioxidant potential at the intracellular level and its involvement in neuroprotection. Cell viability assays enabled us to determine optimal concentrations for each extract (80 µg/mL Tinosporaic acid A and 20 µg/mL B), which were selected on the basis of their cytoprotective actions against H_2O_2, and then tested in the aforementioned oxidative stress marker experiments. In general, our results indicate that both new sterones can partially reverse the H_2O_2-induced deleterious effects on redox status in PC12 cells; in fact, they were able to reduce intracellular ROS formation, and lower lipid peroxidation. More importantly, it seems that they could significantly protect cells from H_2O_2-induced apoptosis.

In conclusion, based on their antioxidative effects, the ethanol extracts of *T. hainanensis*, tested have promising neuroprotective properties, Considered as a whole, our results suggest that *T. hainanensis* could be a good source of natural antioxidant and neuroprotective agents. They merits deserve further investigation, including an exhaustive study of the biological activities of the compounds isolated here.

Conflict of interest statement

The author declares that there is no conflict of interest.

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