ORIGINAL ARTICLE

Investigating apoptotic effects of different extracts of medicinal plants on SH-SY5Y cells

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Abstract

Introduction: Apoptosis, or programmed cell death, occurs naturally during development and has recently gained attention as an important factor in central nervous system disease and injury. **Aim:** The present study was aimed to investigate the apoptotic effects of the different extracts of *Hydrocotyle javanica* (HJ) and *Peristrophe bicalyculata* (PB) on SH-SY5Y Neuroblastoma cells. **Methods and Materials:** The different extracts of HJ and PB were obtained by soxhlation process with petroleum ether, n-hexane, chloroform, alcohol, and water as menstrual. Phytochemical screening was performed for the different extracts of medicinal plants. MTT assay was used to assess the cytotoxicity of the plant extracts, then the induction of apoptosis on SHSY5Y cells by different extracts of HJ and PB was validated by DNA fragmentation analysis using gel electrophoresis technique. **Results and Discussion:** The DNA bands obtained from different extracts of both medicinal plants produced a ladder pattern, as observed from Lane 3 to 10. A ladder formation was used to indicate that the DNA has undergone fragmentation and each fragment corresponded to a band in the ladder. Alcoholic extract of PB showed the fragmentation of DNA at the lowest concentration when compared with other extracts which indicate the potential apoptotic activity at 10 µg/ml. **Conclusion:** According to our findings, Our data well established the antiproliferative effect of different extracts of medicinal plants and clearly showed that the plant extracts can induce apoptosis and not necrosis *in vitro*.

Key words: Apoptosis, gel electrophoresis, *Hydrocotyle javanica*, *Peristrophe bicalyculata*, SH-SY5Y neuroblastoma cells

INTRODUCTION

Apoptosis refers to active, programmed cell death; it is different from necrosis, which is considered to be a passive process. Apoptosis occurs in many forms of central nervous system injury. Both neurons aand glial have been shown to undergo apoptosis after brain and spinal cord injury, stroke, and neurodegenerative diseases. Several factors, including glutamate, cytokines, and free radicals, have been shown to contribute to neuronal and glial apoptosis across different injury mechanisms. Therefore, it is possible that effective inhibitors of apoptosis could be of benefit in several different types of neurological injury.[1]

SH-SY5Y cell is a subline of the SK-N-SH cell line, which was established in culture in 1970 from a bone marrow biopsy of metastatic neuroblastoma of a 4-year-old female and has undergone three rounds of clonal selection.[2] The initial characterization of the SH-SY5Y cell line showed moderate activity of dopamine-β-hydroxylase and negligible levels of choline acetyltransferase, acetylcholinesterase, and butyrylcholinesterase,[2,3] basal noradrenaline (NA) release,[3] and tyrosine hydroxylase activity.[4] Tyrosine hydroxylase is the rate-limiting enzyme of the catecholamine synthesis pathway and converts tyrosine to L-dopa,[5] the precursor of dopamine (DA), which is converted to NA by dopamine-β-hydroxylase.

Hydrocotyle javanica (HJ) Thunb. is a naturally growing prostrate herb found throughout the Himalayas and

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Assam Hills, Nilgiri Hills, and Western Ghats of India at altitudes of 2000–8000 ft.[6] The plant belongs to the family Apiaceae (formerly known as Umbelliferae) and the subfamily is Hydrocotyloideae.[7] Pharmacologically it had been proved that the methanolic fraction of the plant showed antibacterial activity against some human pathogenic bacteria which had been published in our earlier publication.[8]

Peristrophe bicalyculata (PB) is up to 60–180 cm in height and found almost throughout India, Afghanistan, and Africa. It is commonly known as kali aghedi in Hindi and Kakajangha in Sanskrit. The plant belongs to the family Acanthaceae. The chemical composition of the dried aerial parts of PB reveals that it is comprised of 14-methyltritriacont-14-en-15-ol and 35-hydroxynonatriacontanal.[9] The essential oil shows tuberculostatic activity *in vitro* against the growth of various strains of *Mycobacterium tuberculosis*.[9-11]

MATERIALS AND METHODS

Collection and Identification

Collection of two medicinal plants HJ and PB was carried out during the year 2017–2018 on the basis of its medicinal information given by various taxonomists in Pondicherry. A targeted collection based on chemotaxonomic relationships and ethnomedical information derived from traditional medicines. The identified plants were authenticated by Dr. N. Loganathan Specialist in Medicinal plants, Hereditary Physician, Pondicherry.

Preparation of Extract

The collected plant materials were washed with distilled water to remove dirt and soil. The whole plants were further shade dried and then coarsely powdered. The coarse powder of two plants (500 g) was extracted with 3 l of different solvents such as petroleum ether, n-Hexane, chloroform, alcohol, and water (60–80°C) by continuous hot percolation using Soxhlet apparatus. After completion of extraction, it was filtered and the solvent was removed by distillation under the reduced pressure. The extract was stored in a desiccator. The extract was vacuum-dried and processed further for pharmacological evaluation.[12-14]

Phytochemical Screening

The different extracts of HJ and PB were subjected to various chemical tests for identification of phytochemical constituents (glycosides, phytosterol, saponins, alkaloids, carbohydrates, flavonoids, tannins, protein, and amino acid).[15-17]

Apoptosis DNA Fragmentation Analysis Protocol

Principle

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi-quantitative method for confirming apoptosis. DNA cleavage during apoptosis occurs at sites between nucleosomes, protein-containing structures that occur in chromatin at ~200 BP intervals. This DNA fragmentation is often analyzed using agarose gel electrophoresis to demonstrate a "ladder" pattern at ~200 Bp intervals. Necrosis, on the other hand, is characterized by random DNA fragmentation, which forms a "smear" on agarose gels.

Harvest cells

1. Pellet cells, 2. Lyse cells in 0.5 mL detergent buffer: 10 mM Tris (pH 7.4), 5 mM EDTA, 0.2% triton, 3. Vortex, 4. Incubate on ice for 30 min, 5. Centrifuge at 27,000 × g for 30 min, 6. Divide supernatants into two 250 µL aliquots, 7. Add 50 µL ice-cold 5 M NaCl to each aliquot and vortex.

Precipitate DNA

Add 600 µL ethanol and 150 µL 3 M sodium acetate, pH 5.2, and mix by pipetting up and down. Incubate tubes at −80°C for 1 h centrifuge 20,000 × g for 20 min; discard supernatants carefully. Pool DNA extracts together by re-dissolving the pellets in a total of 400 µL extraction buffer (10 mM Tris and 5 mM EDTA). Add 2 µL of 10 mg/ mL DNase-free RNase and incubate for 5 h at 37°C. 6. Add 25 µL proteinase K at 20 mg/mL and 40 µL of buffer (100 mM Tris pH 8.0, 100 mM EDTA, 250 mM NaCl. Incubate overnight at 65°C. Extract DNA with phenol/chloroform/ isoamyl alcohol (25:24:1) and precipitate with ethanol. 8. Carefully discard supernatant trying not to disturb the pellet as it is quite loose.

Load DNA in agarose gel

1. Air-dry pellet and resuspend in 20 µL tris-acetate EDTA buffer supplemented with 2 µL of sample buffer (0.25% bromophenol blue and 30% glycerol).
2. Separate DNA electrophoretically on a 2% agarose gel containing 1 µg/mL ethidium bromide and visualize by ultraviolet transillumination.

Protocol tips

The DNA will make the sample very viscous and sticky. Use the DNA sample loading buffer at a higher concentration than you normally would to ensure the sample does not float away from the well. Prepare an agarose gel with 1.8–2% agarose content. The high agarose concentration provides the necessary resolution to see the steps in the ladder. Run the gel at a lower voltage for a longer time than you normally would to avoid overheating and subsequent deformation of the DNA bands.

Procedure

The monolayer cell culture (SHSY-5Y) was trypsinized and cell count was adjusted to 1 × 105 cells/mL using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. In each well of a 6-well plate, 2 mL of the distilled cell suspension was added and incubated at 37°C for 24 h in an atmosphere of 5% CO_2. After 24 h, when a partial monolayer was formed, the supernatant was discarded and 2 mL of drug dilution prepared in maintenance medium and having concentration below its average, IC_{50} was added to each well in duplicate. The plates were kept for incubation at 37°C in an atmosphere of 5% CO_2. After 24 h of exposure, cells were harvested by trypsinization and centrifuged for 5 min at 3000 rpm. The supernatant was discarded; the cell pellet was dissolved in 1 mL of digestion buffer and 50 µL of proteinase K. The mixture was incubated at 45°C overnight for digestion. Genomic DNA was extracted with an equal volume of chloroform using the standard chloroform extraction procedure. The aqueous phase was separated and treated with 40 µL of RNase at room temperature for 2 h. The impurities were removed with an equal volume of chloroform. After gentle shaking, aqueous phase was separated and the chloroform layer was discarded. DNA was precipitated by the addition of 1–1.5 mL of ice-cold ethanol. The DNA was pelleted by centrifugation at 10,000 rpm for 5 min at 4˚C and the pellet was resuspended in 100 µL of TE buffer. The DNA was dissolved in 100 µL of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 10.0) and electrophoresis of the DNA (at 100 V for 2 h) was carried out on 1.5% agarose gels at 65 V for 45 min or until tracking dye moved to the bottom. The bands were visualized by staining with ethidium bromide.

RESULTS AND DISCUSSION

Phytochemical Screening

The preliminary phytochemical screenings of various extracts of HJ and PB mainly revealed the presence of glycosides,

Table 1: Phytochemical screening of different extracts of *Hydrocotyle javanica*

Phytoconstituents	Petroleum ether	n- Hexane	Chloroform	Alcohol	Aqueous
Alkaloids	–	–	+	+	+
Carbohydrates	–	–	–	+	+
Glycosides	–	+	–	–	–
Phytosterols	+	+	–	+	+
Saponins	–	–	–	–	+
Fixed oils and fats	–	–	–	–	–
Tannin and phenolic compounds	–	–	–	–	+
Proteins and free amino acids	–	–	+	+	+
Gums and mucilage	–	–	–	–	–
Flavonoids	–	–	+	+	+
Lignin	–	–	–	+	+
Volatile oil	–	–	–	–	–

Table 2: Phytochemical screening of different extracts of *Peristrophe bicalyculata*

Phytoconstituents	Petroleum ether	n-Hexane	Chloroform	Alcohol	Aqueous
Alkaloids	–	–	+	+	+
Carbohydrates	–	–	–	+	+
Glycosides	–	+	–	–	–
Phytosterols	+	+	–	+	+
Saponins	–	–	–	+	+
Fixed oils and fats	–	–	–	–	–
Tannin and phenolic compounds	–	–	–	–	+
Proteins and free amino acids	–	–	+	+	+
Gums and mucilage	–	–	–	–	+
Flavonoids	–	–	+	+	+
Lignin	–	–	–	+	+
Volatile oil	–	–	–	–	–

(+) Indicates presence, (–) Indicates absence

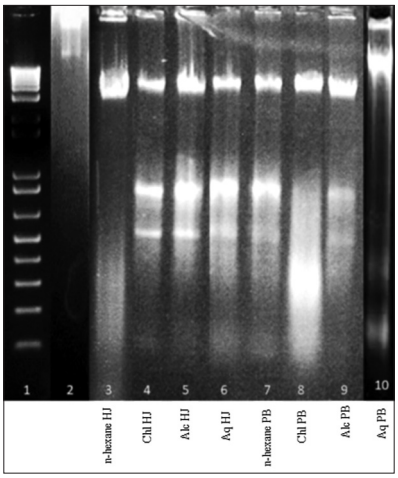

Figure 1: DNA fragmentation assay. Lane 1: DNA ladder, Lane 2: Negative control, Lane 3–10 (sample serial no. 3–10 at a concentration of 10, 20, 20, 50, 50, 40, 10, 20 µg/ml, respectively)

phytosterol, saponins, alkaloids, carbohydrates, flavonoids, tannins, protein, and amino acid in Tables 1 and 2, respectively.

Apoptotic DNA Fragmentation Assay

Induction of Apoptosis on SHSY5Y cells by different extracts of HJ and PB was validated by DNA fragmentation analysis using gel electrophoresis technique. The DNA bands obtained from different extracts of both medicinal plants produced a ladder pattern, as observed from Lane 3 to 10 [Figure 1]. A ladder formation was used to indicate that the DNA has undergone fragmentation, and each fragment corresponded to a band in the ladder. Alcoholic extract of PB showed the fragmentation of DNA at the lowest concentration when compared with other extracts which indicate the potential apoptotic activity at 10 µg/ ml.

CONCLUSION

Our data well established the antiproliferative effect of different extracts of medicinal plants and clearly showed that the plant extracts can induce apoptosis and not necrosis *in vitro*, but its activities remained unknown *in vivo*. These results demonstrated that the different extracts of HJ and PB with antiproliferative properties, especially with IC_{50} value for SHSY5Y cell, might be a novel and attractive therapeutic candidate for neurodegenerative disorder treatment in clinical practice.

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