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Original Research Article

Ameliorative effect of *Vitex peduncularis* in neuroblastoma cells against oxidative stress under hypoxic condition

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ABSTRACT

Vitex peduncularis, a well-known herbal anti-oxidant, has emerged as a potential candidate to ameliorate cellular damage caused due to hypoxia. Formation of reactive oxygen species, malondialdehyde (MDA) and altered level of GSH, GSSG, GPx, GR, and SOD is common during hypoxic stress. N2a cells exposed to hypoxia showed an increase in oxidative damage with enhanced levels of ROS, MDA, GSSG and reduced levels of GSH, GR, and SOD. In the present study, different extracts of *V. peduncularis* i.e. (methanol, chloroform, hexane and benzene) were observed in mitigating oxidative stress in murine neuroblastoma-N2a cell line. The *V. peduncularis* chloroform extract (VPC) was found best in reducing ROS generation. Treatment with VPC significantly restored the levels of GSH, GSSG, SOD and GR in N2a cells under hypoxic conditions. The anti-oxidant activities of VPC were further confirmed by checking expression of anti-oxidative genes, HIF-1α, metallothionine and haemeoxygenase-1 as markers.

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1. Introduction

Hypoxia generally refers to a reduced supply of oxygen to the body. Higher organisms such as human beings are completely dependent on oxygen for their survival and therefore have developed regulatory systems to maintain adequate oxygen homeostasis. When this homeostasis is disturbed under certain conditions, it leads to formation of reactive oxygen species (ROS). People residing at high altitudes generally face this condition. High altitude cerebral oedema (HACE), along with acute mountain sickness (AMS), are major life-threatening complications and may occur in an un-acclimatized person on ascent to high altitudes. AMS is characterized by the presence of headache, nausea, insomnia, dizziness and fatigue (Hackett and Roach, 2001), and is probably due to the formation of mild cerebral oedema. HACE refers to the cerebral abnormalities that occur during high-altitude illness, which is clinically defined as the onset of ataxia, with altered consciousness being considered to be the end stage, eventually leading to death caused by brain herniation (Hackett, 1999). Several studies into the extent of damage and mechanisms of oxidative stress at high altitude are on-going. Oxidative damage to cells has been implicated

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in the pathogenesis of a wide variety of clinical disorders (Halliwell et al., 1987; Droge, 2002), and its broad range of effects in biological systems has led to it being addressed in many experimental studies. Neural tissue is highly sensitive to oxidative stress, which is considered to be a prominent factor in both acute and chronic neurodegenerative diseases, as well as neural insults due to hypoxia.

Nature has provided plants with natural mechanisms to combat oxidative stress by induction or increase in expression of stress-sensitive genes both, locally and systemically (Alvarez et al., 1998). Asada (1999) has also reported that plants have acquired an essential system to reduce and scavenge ROS, which are naturally generated during photosynthesis and respiration (Asada, 1999).

Vitex sp. (family Verbenaceae) are known for their medicinal properties. Approximately ten species from this family are found in India, among which *Vitex negundo* is the most popular. It has been reported to have antipyretic, anti-inflammatory, antibacterial, antiseptic, antitussive, anti-malarial, and analgesic properties (Zuskin et al., 2008). *Vitex peduncularis*, another species from the same genus has not been widely researched for its biological activities. *V. peduncularis* is a tree that grows to be about 18 m tall. It is commonly found in Nepal, Indo-China, Myanmar and Malaysia, and in India it is found in the Eastern Himalayas (Kannathasan et al., 2007).

Iridoids, isolated from the stem bark of *V. peduncularis*, have shown anti-inflammatory activity (Nagarsekar et al., 2010). The chemical constituents of *V. peduncularis* also include alkaloid tri-terpenoids and flavonoids, which are known for their antioxidant properties (Meena et al., 2011). Vitexin, a flavonoid glycoside, that has been isolated from the leaves (0.05%) and root bark (0.07%) has recently shown in animal studies to have both an analgesic and antidepressant-type-effect (Pullaiah, 2006; Borghi et al., 2013; Can et al., 2013). Alcoholic and aqueous extracts of the leaves are reported to possess antibacterial activity against *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* (Kumar et al., 2006).

Hypoxia activates a number of genes, which are important in adaptation to low oxygen conditions at tissue and cellular levels. The cascade of biochemical episodes in hypoxia includes free-radical generation, induction of ROS, cytotoxicity, apoptosis and other damaging events.

Studies have been carried out in order to explore the antioxidant properties of other *Vitex* species such as *V. negundo* (Tiwari and Tripathi, 2007; Devi et al., 2007), whereas, *V. peduncularis*, belonging to same genus, has not yet been tested for its potential in improving the cellular antioxidant system and viability in hypoxia. The larvicidal property of *V. peduncularis* however has been reported (Kannathasan et al., 2007). The authors hypothesized that *V. peduncularis* could be beneficial in restoring cellular oxidative imbalance and improve cell viability in N2a cells under hypoxia. In the present study, four solvents ranging in polarity, i.e. methanol, chloroform, benzene and hexane, were used to prepare the *V. peduncularis* extracts. Six concentrations each of the four extracts (Section 2.2.2) were screened for an improvement in cellular viability by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay and the results further corroborated by FACS (fluorescence activated cell sorting) on the most effective concentration of the extract, i.e. chloroform (VPC) at 125 μg/mL. Hypoxia stress causes imbalance in cellular redox status including lipid peroxidation and excessive ROS leakage, which can be deleterious to cells. Glutathione (GSH)/oxidized glutathione (GSSG) ratio (maintained by glutathione peroxidase i.e. GPx, and glutathione reductase i.e. GR) is an indicative of cellular redox status and decrement in the ratio indicates oxidative damage. Also, hypoxia induces lipid peroxidation malondialdehyde (MDA), excessive reactive oxygen species (ROS) leakage and superoxide generation which lead to cellular damage. Biochemical enzymatic and non-enzymatic redox markers, i.e. GSH, GSSG, ROS, MDA and superoxide dismutase (SOD) can be used as an indicator to assess cellular redox status. Thus, in the present study, *V. peduncularis* extract(s) were used to check its efficacy in providing antioxidant defence under hypoxia by measuring oxidative stress-markers GSH, GSSG, ROS, MDA levels along with measurement GR, GPx and SOD activity.

2. Materials and methods

2.1. *Maintenance of N2a cells*

Murine neuroblastoma (N2a) cells were obtained from the National Centre for Cell Science (NCCS) Pune, India and maintained in the laboratory on DMEM (Dulbecco's Minimal Essential Medium; Sigma) at 37 °C and 5% CO_2 in the CO_2 incubator (Galaxy 170R, New Burnswick). DMEM powder was dissolved in 1 L of sterile distilled water. To this medium, the antibiotics gentamycin sulphate (Sigma; 100 mg/L), streptomycin sulfate (Sigma; 100 mg/L), and sodium salt of ampicillin (Sigma; 100 mg/L) were also added, along with sodium bicarbonate (Sigma; 2.2 mg/L). The pH of the medium was adjusted to 7.2 ± 0.1 using NaOH and HCl. Foetal bovine serum (FBS; 10 mL, Sigma) was added to 90 mL of incomplete medium (10%, v/v) to give a final concentration of 10%. The mixture was then filtered into another sterile medium bottle through syringe filter (Millipore, 0.22 μm), using a 10-mL syringe. The sterility of the medium was checked by keeping the medium at 37 °C in the incubator for 24 h. The sterile medium was kept at 4 °C for storage and further use.

Collection of cells was carried out by de-adherence from the culture flask by trypsinization (0.1% v/v). The cells were counted using Neubauer haemocytometer and seeded in 24-well plates (Nunc, Denmark), with a cell count of ~10^5 cells/well. The N2a culture plates were then incubated in the CO_2 incubator (Galaxy 170R, New Burnswick), maintained at a temperature of 37 °C and 5% CO_2 overnight. The adhered cells were grown to 70–80% confluence. Photographs of cells were taken when grown to confluency using a high-definition microscope (Fluorescent Microscope AE31, Motic, Germany). All photographs were taken at 20× magnification. Hoechst staining of cells under normoxic conditions was carried out to visualize live cells (Fig. 1A–D).

2.2. *Plant material*

V. peduncularis leaves were collected from Tamil Nadu (India) during the months of July–November and the leaves were shade dried. A voucher specimen was sent to

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