Original Article

Substance P inhibitory activity of Azadirachta Indica bark extract-in vitro analysis

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ABSTRACT

Introduction: Substance P is a neuropeptide – but only nominally so, as it is ubiquitous. Through cytoplasmic and nuclear membranes of many cell types of various tissues and organs, its receptor – the neurokinin type 1 – is distributed. Neem bark is taken from the neem tree which predominantly grows in India and Asia. It can be grounded into fine powder and can be used as organic pesticide. It has very strong antibacterial and spermicidal activity. Prior to the advent of toothpaste, the astringent qualities of the bark were used for prevention of bleeding gums, tooth decay and foul smell. Materials and methods: Azadirachta Indica bark extract was obtained from green chem Bangalore. SH-SY5Y neuronal cell lines were obtained from the NCCS, Pune with Passage no 11. Cell culture was maintained at sub confluence in a 95% air, 5% CO2 humidified atmosphere at 37oC using MEM HAM in a 1:1 mixture supplemented with 10% fetal bovine serum supplemented with 100units/ml penicillin and 100µg/ml streptomycin. Differentiation of SH-SY5Y was performed by adding 10µM of all-trans-retinoic acid to the culture medium for 6 days. The differentiated SH-SY5Y cells were incubated with LPS for 2 h. The medium containing the appropriate agents for each condition was replaced every other day. Cells were washed with DMEM + HAM F-12 medium and detached with 0.25% trypsin-EDTA. Results: The short – term exposure of differentiated SH-SY5Y neuronal cells to LPS (1µg/ml) resulted in secretion of inflammatory marker Substance P, indicated by increased level of Substance P in LPS alone induced group. Treatment with different concentrations of neem extract has significantly ($p<0.001$) prevented LPS induced neuro-inflammation in concentrations from 3µg/ml – 100µg/ml. Conclusion: Substance P can be potentially used for pain relief and anti-inflammatory effects.

Keywords: Substance p, neem bark, neuropeptide, inflammatory effects, astringent.

Introduction

Substance P is a peptide which was first described in 1931 [1-6] in extracts of brain and intestine, but which was not purified to homogeneity until 1970 [2,7-14]. The isolation of substance P was accomplished subsequent to the discovery of a sialogogic peptide in hypothalamic extracts [3,15-22], which was shortly thereafter characterised as substance P. The name substance P (for preparation) had been used in the laboratory of origin to designate the active agent in a particular preparation of tissue extracts. This nondescript term entered the literature in 1934 and has persisted [4,23-28]. The amino acid sequence of substance P (SP), H-ARG-PRO-LYS- PRO-GLN-GLN-PHE-PHE-GLY-LEU-MET-NHz, was established in 1971 [5,29-34] and shortly thereafter synthetic peptide was prepared [6,35-40], permitting the development of precise methods for biochemical, histochemical, physiological, and pharmacological studies of the peptide. In addition to the hypotensive and smooth muscle-contracting activity that led to its discovery [1,41-46], SP has many pharmacological effects [7] that are of functional significance [8-14,47]. One likely physiological role for SP is that of a neurotransmitter. Indeed, SP which is widely but selectively distributed in both the central and peripheral nervous systems, is found in fibre tracts and nerve endings, released upon depolarisation, and can alter the activity of some neurones when applied in their vicinity [11, 12,15-18,48].

Substance P is a potent neuroimmunomodulator that functions through lighting members of the neurokinin receptor family one of which, NK1R, is widely expressed in immune cells. Neurokinin type 1 receptor is distributed over cytoplasmic and nuclear membranes of many cell types in many tissues and organs. Neem bark is taken from the neem tree which

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predominantly grows in India and Asia. It can be grounded into fine powder and can be used as organic pesticide. It has very strong antibacterial and spermicidal activity. It is traditionally used to treat tiredness, fever and loss of appetite [13,19,20,49,50].

Medicinal plants are an important part of human society to overcome various diseases, from the dawn of civilisation. Azadirachta indica (Neem) is one of them which is well known in India and its neighbouring countries for more than 2000 years. It is one of the most versatile plants having wide biological spectrum. All the parts of the tree are used as traditional medicine in household [14, 15,21,22]. In ayurveda Neem is used in Leprosy, eye problem, epistaxis, intestinal worms, anorexia, biliousness, and skin ulcers. Its Bark is used as analgesic, alternative and curative of fever. Flower is used for elimination of intestinal worms and phlegm. Fruit Relieves piles, intestinal worms, urinary disorder, epistaxis, eye problem, diabetes, wounds and leprosy. Gum is Effective against skin diseases like ringworms, scabies, wounds and ulcers [16,51-58]. Neem is a member of Meliaceae family [13, 17,23,24,59]. The importance of the neem tree has been recognised by the US National Academy of Sciences, which published a report in 1992 entitled 'Neem – a tree for solving global problems' [15, 17,60-64]. Bark, leaf, root, flower and fruit together cure various diseases like blood morbidity, biliary afflictions, itching, skin ulcers, and burning sensation [18]. It protects the crop, grains and clothes from the attack of insects [19, 20,25,26]. The compounds of neem have been divided into two major classes isoprenoids and other. Isoprenoids is again divided into diterpenoids and triterpenoids [21-23,27-32]. Neem has been extensively used in ayurveda, homeopathy and unani medicine which has become a cynosure of modern medicine [15, 24,33-36].

Hence this study aims to do a research on substance P inhibitory activity of neem bark.

Materials and Methods

Azadirachta Indica bark extract was obtained from green chem Bangalore.

Chemicals

Lipopolysaccharide (LPS), Phenol free Dulbecco's modified Eagle medium (DMEM) + Ham's F12 media, Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100µg streptomycin, and 0.25µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen.

Cell culture

SH-SY5Y neuronal cell lines were obtained from the NCCS, Pune with Passage no 11. Cell culture was maintained at sub confluence in a 95% air, 5% CO2 humidified atmosphere at 37°C using Eagle's Minimum Essential Medium (MEM) and Ham's Nutrient Mixture F12 (HAM) in a 1:1 mixture supplemented with 10% fetal bovine serum supplemented with 100units/ml penicillin and 100µg/ml streptomycin. Differentiation of SH-SY5Y was performed by adding 10µM of all-trans-retinoic acid to the culture medium for 6 days [37]. The differentiated SH-SY5Y cells were incubated with LPS (1µg/mL) for 2 h. The medium containing the appropriate agents for each condition was replaced every other day. Cells were washed with DMEM + HAM F-12 medium and detached with 0.25% trypsin-EDTA.

Inhibitory activity of Neem extract against Substance P

Substance P is a neuropeptide implicated in the etiopathology of neurogenic inflammation, depression and anxiety [26]. The differentiated SH-SY5Y cells were treated with different concentrations (1, 3, 10, 30 and 100µg/ml) of neem extract for 20h. At the end of incubation period, LPS (1µg/mL) was added and further incubated for 2 h. To estimate the amount of Substance P, the extract + LPS treated cells were scraped and diluted to the cell concentration of approximately 1 million/ml. The cells were damaged through repeated freeze-thaw cycles to let out the inside components. Then it was centrifuged at 2000-3000rpm for approximately 20 minutes. The supernatant was collected and estimated for Substance P by ELISA kit analysis.

Results

The short – term exposure of differentiated SH-SY5Y neuronal cells to LPS (1µg/ml) resulted in secretion of inflammatory marker Substance P, indicated by increased level of Substance P in LPS alone induced group. Treatment with different concentrations of neem extract has significantly ($p<0.001$) prevented LPS induced neuro-inflammation in concentrations from 3µg/ml – 100µg/ml as seen in Figure 1.

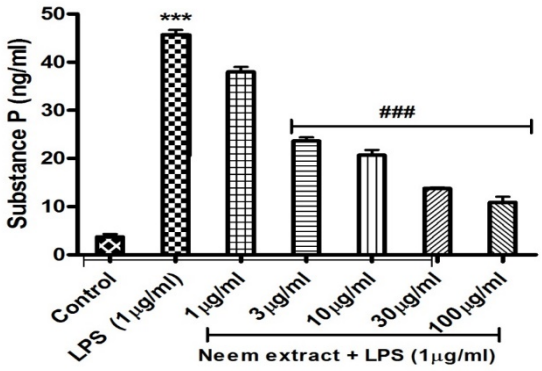

Figure 1

Values are expressed as Mean ± SEM. ***P<0.001 considered significant Control Vs. LPS; ###P<0.001 considered significant LPS Vs. neem extract (Figure 1).

Discussion

Substance P, indicated by increased level of Substance P in LPS alone induced group. Treatment with different concentrations of neem extract has significantly ($p<0.001$) prevented LPS induced neuro-inflammation in concentrations from 3µg/ml – 100µg/ml as shown in Figure 1.

Pain induced by thermal stimulus of the analgesiometer is specific for testing centrally mediated analgesic activity. Opioid agents (Morphine) acts via supra spinal (µ1, κ3, σ2, δ1) and spinal (µ2, κ1, δ2) receptors. These endogenous peptides thereafter descend the spinal cord and inhibit the pain impulse transmission at the synapse in the dorsal horn. The possible mechanism of Neem bark could be due to its action on the central opioid receptors or through release of endogenous opioid peptides [38]. Combination of morphine and neem leaf extract produce greater analgesia with lesser side effect [16].

Analgesic activity of neem root bark extract in both peripheral and central mechanism, comparison to standard drugs it was

having very weak analgesic action. Neem root bark contains terpanoids like nimbin and nimbidin.

The reason for weak analgesic activity may be quantity of nimbidin which is present in root bark. Other chemical constituents may also be responsible for analgesic activity.

Previous studies have shown that neem has anti-inflammatory, antipyretic and analgesic activities. The chloroform extract of stem bark is effective against carrageenan induced rat paw oedema. Inflammatory stomatitis in children is cured by the bark extract. Antipyretic activity has also been reported in neem oil [17].

Conclusion

In summary, inhibition of substance P has been demonstrated or proposed to be the important neurochemical event through which presently available analgesic agents successfully relieve oral pain. Pharmaceutical companies realising these basic pharmacological mechanisms ofactionare feverishly attempting to develop substance P receptor antagonists which will selectively and completely block oral pain. Very recent reports of substance P antagonists have already appeared and may well prove to be the dawning of a new era in the successful management of oral pain[].

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