RESEARCH ARTICLE

Preliminary phytochemical screening and *in vitro* anti-inflammatory activity of *Azadirachta indica* leaves by human red blood cells

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ABSTRACT

Azadirachta indica (A. indica) commonly known as Neem belongs to the family Meliaceae. This study was aimed to investigate the *in vitro* anti-inflammatory activity of ethanol extract from *A. indica* leaves (EAL). EAL was investigated for the presence of phytochemicals and *in vitro* anti-inflammatory activity in human red blood cell membrane stabilization (HRBC) method. Diclofenac sodium was used as the standard drug for *in vitro* anti-inflammatory activity. On the basis of qualitative analysis of ethanol extract of leaves of *A. indica* showed the presence of carbohydrate, glycoside, flavonoids, alkaloids, tannin, and phenolic compounds as secondary metabolites. Furthermore, ethanol extract of leaves of *A. indica* and diclofenac sodium showed a concentration dependent stabilization toward HRBC membrane with 298.7 ± 0.017 and 34.98 ± 0.012 µg/ml; 50% protection, respectively. EAL possessed noticeable *in vitro* anti-inflammatory effect against the HRBC membrane stabilization method. Further, commanding studies are essential to make certain the mechanisms and constituents last its anti-inflammatory actions.

Keywords: *Azadirachta indica,* human red blood cell membrane stabilization, *in vitro* anti-inflammatory model

Introduction

Herbal and natural products of folk medicines have been used for centuries in every culture worldwide.[1] Scientists and medical professionals have shown increased interest in the field as they recognized the true health benefits of these remedies.[2] One of the characteristics of oriental herbal medicine preparations is that all the herbal medicines, either presenting as single herbs or as collections of herbs in composite formulae.[3] Natural products, in general, and medicinal plants, in particular, are believed to be an important source of new chemical substances with potential therapeutic efficacy. Medicinal plants are the basic for the treatment of various diseases.

Plants are being used in medicine from time immemorial because they have fitted the immediate personal need, they are accessible and inexpensive, the practitioners speak to those who have used them in their own language, and they are not provided from a remote professional or government apparatus. For these and other reasons, the use of plants for medicines worldwide still vastly exceeds the use of modern synthetic drugs. Such activity is not completely dismissed in scientific society and plants are also appreciated in pharmaceutical research as the major resource for new medicines, and a growing body of medical literature supports the clinical efficacy of herbal treatments.[4,5] Even where traditional use has largely died out in developed countries, there is an increasing yearning for a new deal in health care in which the old remedies feature strongly. In India, the herbal drug market is about $ one billion, and the export of plant-based crude drugs is around $ 80 million.[6] The World Health Organization estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs for their primary health care needs. Even the modern pharmacopeia still contains at least 25% drugs derived from plants and many others

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which are semi-synthetic, built on prototype compounds isolated from plants.[7]

Inflammation is characterized clinically by signs such as edema (swelling), tenderness, and pain. Prostaglandins and histamines have been implicated in these inflammatory processes. Inflammatory responses occur in three different phases each phase is mediated by different mechanism. An acute, transient phase is characterized by local vasodilatation and increased permeability. A subacute phase is characterized by infiltration of leukocytes and phagocyte cell. A chronic proliferative phase, in which tissue degeneration and fibrosis occurs. Inflammation is a path psychological response of living tissue to injury that lead to the local accumulation of plasmatic fluid and blood cells. Inflammation is a path physiological response of living tissues to injuries that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases.[8] In general, the process of inflammation is associated with the activation and involvement of secretion of cytokines such as IL-1β, IL-6, and TNF-α, by activated cells which play major role in host defense mechanisms. The use of steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) have been used for rapid and effective for only temporary relaxation and are inadequate.[9]

Azadirachta indica which commonly known as Neem belongs to the family of Meliaceae and has been used in Ayurvedic treatment for more than 4000 years ago. Neem is native to east India and Burma and grows much in South East Asia (SEA) and West Africa. Furthermore, Neem is cultivated in Pakistan, Singapore, Philippines, and Australia.[10] Neem may grow up to 50 feet (15 m) tall and live for 200 years. The lifespan of the Neem tree is described to be anywhere between 150 and 300 years. Neem is evergreen but can shed most of its leaves under dry conditions. Neem trees can grow in areas having rain each year between 400 mm and 1500 mm. It grows best at an altitude of <1,500 m. Neem trees survive at very hot temperatures, up to 44°C and down to 4°C. Some people reported neem trees surviving in light frost.[11]

Morphological description

Leaves are compound, alternate, rachis 15-25 cm long, 0.1 cm thick; leaflets with oblique base, opposite, estipulate, lanceolate, acute, serrate, 7-8.5 cm long and 1.0-1.7 cm wide, slightly yellowish-green and bitter in taste. Stem Bark: Stem bark varies much in thickness according to age and parts of tree from where it is taken; external surface rough, fissured and rusty-gray; laminated inner surface yellowish and foliaceous, fracture, fibrous; characteristic; taste, bitter.[12] Flowers: The flowers (white and fragrant) are arranged axillary, normally more-or-less drooping panicles which are up to 25 cm long. The flowers are 4-7 mm in length and 6-10 mm in width.[13] Ripe fruit of neem is about 2 cm long and oval shaped. Inside the fruit there is a light colored seed about 1.5 cm long.[11] Chemical constituents: A large number of biologically active compounds are present in *A. indica* including azadirone, promeliacin, limonoids, gedunin, vilasinin, C-secomeliacins, azodirachtin, nimbin, salanin and other non-iosprenoids, as well as protein/amino acids, polysaccharides, sulfurous compounds, polyphenolics such as flavonoids, their glycosides, dihydrochalcone, coumarins, tannins, and aliphatic compounds.[14] Nimbidin and Azadirachtin are isolated from the seed of the neem tree.[15]

Almost all parts of the neem tree have been used as traditional Ayurvedic, Unani, and Sidhha medicine in India. Neem oil, bark, and leaf extracts have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, constipation and blood purifier along with general health tonic. It is also used for the treatment of rheumatism, chronic syphilitic sores and indolent ulcer. Neem.[5] Leaves, bark, root, and oils also used as insecticidal, good in opthalmintic condition, antiperiodic, vermifuge stomachic, snakes bites piles, and wound healing.[16] Neem seed are also ued as antidiabetic, antibacterial.[5] Neem also used in oxidative stress, inflammation, arthritis.[17] Various pharmacological actions of neem are also present such that they can be used as antioxidant activity,[18] central nervous system depressant activity,[19] abrotifacient activity,[20] antidiabetic activity,[21] antifertility activity,[22] hepatoprotective activity,[23] antiseptic, wound healing and skin disease curing activity,[24,25] hypoglycemic and antihyperglycemic activity.[26] This study was aimed to carry out preliminary phytochemical screening and evaluate *in vitro* anti-inflammatory activity of *A. indica* leaves by human red blood cells (HRBC).

Materials and Methods

Collection and authentication of plant

The leaves of *A. indica* were collected from the Moradabad in the month of February. The plant was authenticated by Dr. Ashok Kumar, Assistant Professor, and a reputed botanist. The fresh leaves of *A. indica* were collected and dried under shade for 15-20 days and homogenized to get a coarse powder. This powder was stored in an air tight container for further use.

Chemicals and reagents

Chemicals used were obtained from Central Drug House Pvt. Ltd., New Delhi; Fischer Scientific Laboratories, Mumbai. A Diclofenac drug as a gift sample procured from CIPLA, Baddi, Himachal Pradesh, India.

Preparation of plant extracts

About 400 g powdered leaves of *A. indica* was defatted with petroleum ether, and subsequently extracted with ethanol. The ethanol extract was concentrated at reduced temperature and pressure using a rotary evaporator. A yield for ethanol extract of leaves of *A. indica* (EAL) was found as 4.5% (w/w).

Preliminary phytochemical screening

Preliminary phytochemical screening of EAL was carried for their contents of different classes of compounds. The qualitative chemical tests for various phytoconstituents were carried out as per standard procedures.[27]

In vitro anti-inflammatory activity

Ethanol extract was further investigated for anti-inflammatory activity through HRBC membrane stabilization method as *in vitro* models. *In vitro* anti-inflammatory activity was extensively studied by the HRBC membrane stabilization method.[28,29] In this; blood was collected from a human volunteer who had not taken any NSAIDs for 2 weeks before this experiment and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl). The resulting volume was centrifuged at 3000 rpm. The packed cells were washed with isosaline (0.9% w/v NaCl), and a 10% suspension was made. The reaction mixtures consist of the ethanol extract with different concentration (25, 50, 100, 200, 400, and 800 µg/ml) and to each concentration 1 ml phosphate buffer, 2 ml hyposaline, and 0.5 ml HRBC suspensions were added. These were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin content in the supernatant solution was estimated spectrophotometrically in triplicates at 560 nm. Diclofenac sodium with different concentration (10, 20, 40, 60, 80, and 100 µg/ml) was used as the reference standard and a control was prepared without the extract or standard. The percentage hemolysis was calculated by assuming the hemolysis produced within the control group as 100%. The percentage of HRBC membrane stabilization or protection was calculated using the formula:

$$\text{Percentage protection} = \frac{\text{Absorbance of control-absorbance of test}}{\text{Absorbance of control}} \times 100$$

Result

Preliminary phytochemical screening

On the basis of qualitative analysis of ethanol extract of leaves of *A. indica* showed the presence of carbohydrate, glycoside, flavonoids, alkaloids, tannin, and phenolic compounds as secondary metabolites.

In vitro anti-inflammatory activity

The result from the HRBC membrane stabilization method was shown in Figures 1 and 2. The EAL showed a concentration dependent anti-inflammatory activity, and the protection percent increased by an increase as the concentration of the EAL. From the dose-response curve, plotted as log [EAL (µg/ml)] against percentage response keeping the hemolysis produced within the control group as 100%, 50% percentage response were found at 298.7 ± 0.017 µg/ml and similarly, for diclofenac sodium it was 34.98 ± 0.012 µg/ml.

Discussion

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body's response to inactivate or destroy the invading organisms, to remove the irritants, and set the stage for tissue repair.[30] It is triggered by liberating chemical mediators or signaling molecules from injured tissue and migrating cells. The mechanism of inflammation is attributed, in part, to release of reactive oxygen species from

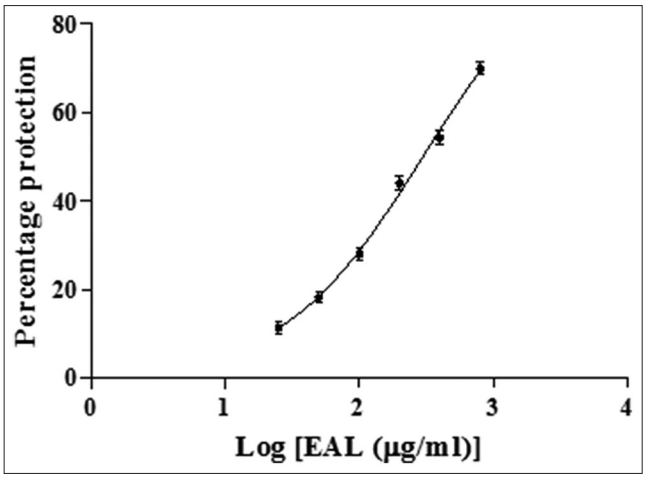

Figure 1: Dose response curve for ethanol extract from *Azadirachta indica* leaves in human red blood cell membrane stabilization method (n=3)

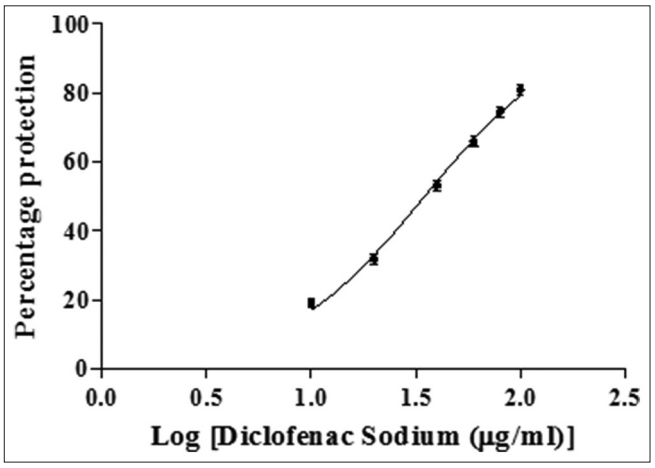

Figure 2: Dose response curve for diclofenac sodium in human red blood cell membrane stabilization method (n=3)

activated neutrophil and macrophages. This overproduction leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes.[31,32]

The EAL exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogs to the lysosomal membrane, and its stabilization implies that the EAL may as well stabilize lysosomal membrane. Injury to lysosomal membrane usually triggers the release of phospholipase A_2 that mediate the hydrolysis of phospholipids to produce inflammatory mediators.[1] Stabilization of the membranes from these cells inhibits lysis and subsequent release of the cytoplasmic contents which in turn limits the tissue damage and exacerbation of the inflammatory response.[33] This might have contributed to the anti-inflammatory activity of the EAL.

Conclusions

On the basis of qualitative analysis of ethanol extract of leaves of *A. indica* showed the presence of carbohydrate, glycoside, flavonoids,

alkaloids, tannin, and phenolic compounds as secondary metabolites. Furthermore, it can be resulted that EAL possessed noticeable *in vitro* anti-inflammatory effect against the HRBC membrane stabilization method. Further authoritative studies are necessary to make certain to the mechanism and constituents behind its anti-inflammatory actions.

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