POTENTIAL IMMUNOMODULATING ACTIVITY OF NEEM (*AZADIRACHTA INDICA*) LEAF EXTRACTS AND ITS BIOACTIVE COMPOUND N-ACETYLGLUCOSAMINE IN MICE

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by

VENUGOPALAN SANTHOSH KUMAR

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DEDICATION

To His Holiness Melmaruvathur Arulthiru Amma and family
And
To my Parents Mr. M. Venugopalan
(Retired Higher Secondary School Head Master)
Mrs. N. Balagujambal
(Retired Higher Secondary School Teacher)

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LIST OF ABBREVIATIONS & SYMBOLS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
DEX	Dexamethasone
D_2O	Deuterium Oxide
DMSO	Dimethyl sulfoxide
ED_{50}	Effective dose 50%
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
g	Gram
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferases
h	Hour
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
IL-2	Interleukin-2
i.p.	Intraperitoneal
α- KDH	alpha ketoglutarate dehydrogenase
L	Litre
LD_{50}	Lethal dose 50%
LPO	Lipid peroxide
µg	Microgram
MDA	Malondialdehyde
MDH	Malate dehydrogenase
mg	Milligram
min	Minute
ml	Millilitre
MnSOD	Mitochondrial antioxidant manganese superoxide dismutase
MW	Molecular Weight
NADPH	Nicotinamide adenine dinucleotide phosphate

NAG	N-Acetylglucosamine
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NK cells	Natural killer cells
NMR	Nuclear magnetic resonance
O-NAG	O-linked N-Acetylglucosamine
PBS	Phosphate buffer saline
pg	Pico Gram
ROS	Reactive oxygen species
ROO	Peroxyl radical
R_f	Retention factor
SD	Standard deviation
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase
RPM	Rotation per minute
temp.	Temperature
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TMB	3, 3', 5', 5' – Tetramethylbenzidine
UV	Ultra violet
UNEP	United Nations Environment Programme
WA	Withanolide A
wt	Weight
WHO	World Health Organization

POTENSI AKTIVITI IMMUNOMODULASI BAGI EKSTRAK DAUN NEEM (*AZADIRACHTA INDICA*) DAN SEBATIAN BIOAKTIFNYA N-ACETYLGLUCOSAMINE DALAM MENCIT

ABSTRAK

Kajian ini bertujan untuk menilai potensi aktiviti immunostimulan dari daun *Azadirachta indica* N-Acetylglucosamine (AILG) dalam mencit. Ekstrak heksana, klorofom, metanol dan akueus daun *A. indica* telah disediakan dan aktiviti immunostimulan telah dikaji. Ekstrak akueus daun *A. indica* (AEAIL) menunjukkan aktiviti immuostimulan yang lebih signifikan ($P<0.001$) daripada lain-lain ekstrak. Oleh itu pengasingan juzuk fito dari AEAIL telah dijalankan dan N-Acetylglucosamine (NAG) telah di pencilkan. NAG telah diberikan pada 266, 400, 800 μg / kg kepada mencit secara intraperitonial selama 4 minggu untuk menilai aktiviti immunostimulan. Paras serum interleukin-2 (IL-2) dan kajian histopatologikal pada timus telah dilakukan untuk mengesahkan aktiviti immunostimulan (NAG). Pemberian NAG dalam dos seperti di atas meningkatkan serum IL-2 secara signifikan ($P<0.001$) dalam mencit ujian berbanding mencit kawalan. Kesan pergantungan dos bagi IL-2 telah diperhati dalam mencit yang dirawat dengan NAG. Berat timus, hati dan buah pinggang meningkat secara signifikan ($P<0.001$) dari minggu kedua ke minggu keempat berbanding mencit kawalan. Percambahan T-limfosit dalam timus selepas pemberian NAG diperhatikan dalam kajian histopatologikal. Kesan NAG dan withanolide A (WA - kawalan positif) pada penanda mitokondria, sistem antioksida, perubahan ultrastruktur dalam mitokondria, nukleus dan kajian histopatologi telah dijalankan dalam mencit teraruh deksametason (DEX). Pendedahan kepada DEX mengurangkan paras penanda mitokondria, enzim antioksida, adenosina trifosfat (ATP) dan peningkatan paras lipid

peroksida (LPO) berbanding kumpulan, kawalan. Rawatan dengan NAG (266, 400, 800 μg / kg) dan WA (800 μg / kg) menunjukkan bahawa terdapat peningkatan yang ketara dalam paras penanda mitokondria dan enzim antioksida, ATP dan paras LPO menurun berbanding kumpulan yang dirawat dengan DEX. Kajian mikroskop transmisi elektron (TEM) mendedahkan bahawa kumpulan yang dirawat dengan DEX menunjukkan perubahan yang merosakkan kristae mitokondria dan nukleus bengkak piknotik manakala kumpulan yang dirawat dengkan NAG menunjukkan penyusunan semula pembentukan kristae mitokondria dan nukleus. Kajian histopatologi menunjukkan bahawa kumpulan timus tikus yang dirawat dengkan NAG menunjukkan percambahan timosit berbanding kumpulan kawalan. Mekanisme yang munasabah mengenai NAG boleh dikaitkan dengan membaik pulih enzim mitokondria maka dengan itu meningkatkan pengeluaran tenaga mitokondria yang membawa kepada peningkatan IL-2 dan ekspresi IL-2R dalam timus mencit.

POTENTIAL IMMUNOMODULATING ACTIVITY OF NEEM (*AZADIRACHTA INDICA*) LEAF EXTRACTS AND ITS BIOACTIVE COMPOUND N-ACETYLGLUCOSAMINE IN MICE

ABSTRACT

Present study was aimed to evaluate the potential immunostimulant activity of N-Acetylglucosamine (NAG) from *Azadirachta indica* leaves in mice. The hexane, chloroform, methanol and aqueous extracts of *A. indica* leaves were prepared and its immunostimulant activity was studied. The aqueous extract of *A. indica* leaves (AEAIL) showed significant ($P< 0.001$) higher immunostimulant activity than other extracts. Hence, isolation of possible phytoconstituent(s) from AEAIL was carried out and the N-Acetylglucosamine (NAG) was isolated. The NAG was administered at 266, 400 and 800 µg/kg of mice, intraperitoneal route weekly for 4 weeks to evaluate immunostimulant activity. The serum interleukin-2 (IL-2) level and histopathological studies on thymus were performed to confirm NAG immunostimulant activity. The administration of above doses of NAG has significantly ($P< 0.001$) increased serum IL-2 levels in mice than control mice. The dose dependent effect on IL-2 was noticed in NAG treated mice. The weight of thymus, liver and kidney were significantly ($P< 0.001$) increased after the NAG treatments compared to control mice. Also, body weight of NAG treated mice showed significant ($P< 0.001$) increment from second week to fourth week than control mice. The proliferation of T-lymphocytes in thymus after the administration of NAG was observed in histopathological study. Effect of NAG and withanolide A

(WA - positive control) on mitochondrial markers, antioxidant system, ultrastructural changes in mitochondria, nucleus and histopathological studies were performed in dexamethasone induced thymus of mice. Exposure to dexamethasone (DEX) leads to decreased level of mitochondrial markers, enzymatic antioxidants, adenosine triphosphate (ATP) and increased level of lipid peroxide (LPO) when compared to control group. Treatment with NAG (266, 400, 800 µg/kg) and WA (800 µg/kg) showed that there was a significant increase in the levels of mitochondrial markers and enzymatic antioxidants, ATP and decreased level of LPO when compared to DEX treated group. Transmission electron microscopy (TEM) studies revealed that DEX treated group shows destructive alterations of mitochondrial cristae and pyknotic swollen nucleus whereas NAG treated group shows the restructuring of the formation of cristae of mitochondria and nucleus. Histopathological studies showed that NAG treated group mice thymus shows proliferation of thymocytes when compared to control group. The plausible mechanism of NAG was associated with the restoration of mitochondrial enzymes thereby improving mitochondrial energy production that leads to elevation of IL-2 and expression of IL-2R in thymus.

CHAPTER 1

INTRODUCTION

1.1 Background of study

The United Nations Environment Programme announced neem as a "Tree of the 21st century". In 1992 the US National Academy of Science tabled a report mentioning Neem as the tree for solving global problems". Immunosupression is a decrease in immune function measured as an effect on cellular, humoral, or non-specific immune parameters. The likely clinical sequels of immunosupression are increased rates of infectious diseases and neoplasia. Clinicians agree that susceptible groups are more likely to suffer adverse health consequences from any immune suppression.

The immune system and its response to stimulation can be disrupted by oxidative stress and this may be hugely due to underlying immune dysfunction (Sordillo and Aitken, 2009). The thymus is a primary lymphoid organ contributing immune longevity. When there is impaired immunity, the individual suffers from increased susceptibility to a variety of illnesses due to dysfunction of thymocytes (Dietert and Zelikoff, 2010; Morris et al., 2013). It has become clear that oxidative stress is the key factor responsible for the onset and progression of a number of immunological disorders (Hybertson et al., 2011; Daniela et al., 2009). It is also well accepted now that apoptosis induced by the oxidative stress in thymocytes is an imperative for immunosuppressive function in human and various animal species *in vivo* and *in vitro* (Kumar et al., 2015; Yi et al., 2016). There is clear evidence that loss of function in

the respiratory chain complex in mitochondria leads to apoptosis and thymocyte death (Ricci et al., 2003).

Scientists are now able to mass produce immune cell secretions, both antibodies and lymphokines, as well as specialized immune cells. The ready supply of these materials not only has revolutionized the study of the immune system itself but also has had an enormous impact on medicine. Medicinal plants have been used as sources of medicine for boosting the immune system with less adverse events. They have been investigated for immunomodulatory potentials and it has been proved by diverse mechanisms in animal models that medicinal plants have beneficial effect on the immune system. The use of immunostimulants has opened a very promising new chapter in immunotherapy. Immunostimulants are used to enhance the bodys immune system and play a vital role to treat immune deficiencies.

1.1.1 Neem tree plant sources

From prehistorical times the neem tree (*Azadirachta indica*) has been considered a divine tree. It was during the Indus Valley Civilization that the first medicinal uses of the neem plant were recorded. Neem is considered as a blessed tree (Figure 1.1). It belongs to the Family Meliaceae, the mahogany family, and the botanincal name of neem is *A. indica* A. Juss (Table 1.1). The name *Azadirachta indica* is derived from the Persian language where *Azad* means “free”, *dirakht* means “tree” and *i-Hind* means “of Indian origin”. In other words, it can be called “The Free Tree of India”.

Table 1.1 Taxonomic position of *A. indica* (Girish and Shankara, 2008).

ORDER	RUTALES
Family	Meliaceae
Genus	*Azadirachta*
species	*indica*

Figure 1.1 Neem Tree (Girish and Shankara, 2008).

1.1.2 Local names of neem

Neem is native to the Indian subcontinent. The names of neem in various Indian and world languages are listed in Table 1.2 (Imam, Hussain and Ajij, 2012). From time immemorial, medicinal plants have been used for the treatment of many infectious diseases without any scientific evidence. At present there is more emphasis on determining the scientific evidence and rationalization of the use of these preparations.

Table 1.2 Local names of neem

Languages	Local names of neem
English	Neem
German	*Indischer Zadrach*
Persian	*Azade Darakhte Hindi*
Malay	*Pokok Semambu, Dawoon Nambu, Baypay*
Chinese	*Lian shu, Lian zao zi, Yin du lian shu*
Arabic	*Azad Darkhtu Hind*
Thai	*Khwinin, Sadao India*
Burmese	*Tamabin, Kamakha*
Latin	*Azadirachta indica A. Juss or Melia azadirachta Linn*
Singapore	*Nimba*
French	*Azarirae d'lnde, Margousier*
Indonesia	*Mimba*
Nigeria	*Don goyaro*
Simhalee	*Nimu*
Spanish	*Margosa*
Indonesia	*Mimba*
Portuguese	*Margosa, Nimbo*
Tamil	*Vembu*
Hindi	*Neem*
Marathi	*Kadunimb*
Gujarati	*Leemdo*
Punjabi	*Nimb*
Malayalam	*Veppu, Aryaveppu*
Telegu	*Vepa*
Kannada	*Bevinmar, Kahibevu*

1.2 Literature review

The neem tree *(A. indica)* is mainly cultivated in South East Asia. Quality assurance of raw materials of neem and processing of raw materials and traditional uses of neem were well documented (Puri, 1999). The neem tree has been used as a source of unique natural products for integrated pest management, medicine, industry and other purposes (Schmutterer, 1995). Neem root, bark and young fruit have astringent, tonic and antiperiodic activity. Bark is bitter, vermifuge and cures ulcers. Neem leaves have anthelmintic, insecticidal activity and can be used to treat skin diseases (Upma et al., 2011). In traditional Indian medicine neem plays a major role in the treatment of various human diseases. Numerous biologically active compounds were present in neem and recognized for various pharmacological effects (Sushma, Smriti and Sheveta, 2012). Historical survey of indigenous drugs and the evolution of the present Indian indigenous drugs were documented. In the rural areas of India water extraction of neem leaves is used for the prophylactic and therapeutic treatment of various diseases. Medicinal uses of various parts of neem are listed in Table 1.3.

Botelho et al. (2008) studied the efficacy of a neem leaves based mouth rinse. The aim of the study was to compare the short term efficacy and safety of a *A. indica* mouth rinse on gingival inflammation and microbial plaque, compared to 0.12% chlorhexidine. A double - masked, randomized, parallel armed study was carried out to assess the efficacy of the neem leaves based oral mouthrinse in reducing gingivitis.

Biswas et al. (2002) studied the biological activities and medicinal properties of neem and reported that neem leaf, bark, flowers, fruit, twig, gum, oil and root has been used

to treat leprosy, piles, cough and ring worms. Neem (*A. indica*) is perhaps the most useful traditional medicinal plant in India. Each part of the neem tree has some medicinal property and is thus commercially exploitable. This review gives a bird's eye view mainly on the biological activities of some of the neem compounds isolated, pharmacological actions of the neem extracts, clinical studies and plausible medicinal applications of neem along with their safety evaluation.

Table 1.3 Medicinal uses of neem (Biswas et al., 2002).

Part	Medicinal use
Leaf	Leprosy, eye problem, epistaxis, intestinal worms, anorexia, biliousness, skin ulcers.
Bark	Analgesic, alternative and curative of fever.
Flower	Bile suppression, elimination of intestinal worms and phlegm.
Fruit	Relieves piles, intestinal worms, urinary disorder, epistaxis, phlegm, eye problem, diabetes, wounds and leprosy.
Twig	Relieves cough, asthma, piles, phantom tumour, intestinal worms, spermatorrhoea, obstinate urinary disorder, diabetes.
Gum	Effective against diseases like ring worms, scabies, wounds and ulcers.
Seed pulp	Leprosy and intestinal worms.
Oil	Leprosy and intestinal worms.
Root, bark, leaf, flower and fruit together	Blood morbidity, biliary afflictions, itching, skin ulcer, burning sensation and leprosy.

1.2.1 Neem in human health

1.2.1 (a) First aid

Neem's antiseptic and healing properties make it an excellent first aid for minor cuts and abrasions, and it has been shown to be an excellent wound healer. Neem has the ability to increase vascular permeability by increasing the blood flow and by helping

the body to rapidly create collagen fibers to close wounds.There are reports from the National University of Singapore (Jin et al., 2013) stating that tissue engineered plant extracts can be used as nanofibrous wound dressings.

1.2.1 (b) Antibacterial activity

Anti-microbial activities of aqueous extracts from neem were reported by Wafaa, Hassan and Nefisa (2007). The aqueous extraction of neem leaves, seeds and seed hulls was carried out at 80°C under acidic, neutral and alkaline conditions. The sulphation of nine crude extracts was carried out using chlorosulphonic acid as sulfating agent in formamide. Anticoagulation and fibrinolytic activities, as well as antimicrobial effect of aqueous extracts and their corresponding sulphates are demonstrated. Native extracts and their corresponding sulfates gave various inhibition activities against the gram positive bacteria *Staphylococcus aureus*, the gram negative bacteria *Escherichia coli*, the yeasts *Candida albicans*, Fungi *Aspergillus niger* and *Penicillium citrinum.*

Yu et al. (2010) isolated the antibacterial compound from petroleum ether extract of neem oil. The tetrahydrofuranyl diester from neem oil was investigated for antibacterial activity against three bacterial strains *viz., S. aureus, Escherichia coli* and *Salmonella* sp. Pu et al. (2010) studied the antibacterial activity of 9-octadecanoic acid-hexadecanoic acid-tetrahydrofuran-3,4-diyl ester from neem oil. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the 9-octadecanoic acid-hexadecanoic acid-tetrahydrofuran-3,4-diyl ester were determined by using the broth microdilution (BMD) method at different concentrations ranging from 20 to 0.625 mg mL^{-1}. The

antibacterial activity of 9-octadecanoic acid-hexadecanoic acid-tetrahydrofuran-3,4-diyl ester against the three strains tested showed the relationship with time and concentration.

1.2.1 (c) Antiviral

In vitro antiviral property of neem against poliovirus was reported by Ligia (2012). Study aims at evaluating the activity of a polysaccharide (P1) such as water soluble pectic arabinogalactan isolated from the leaves of *A. indica* and their chemical sulfated derivatives (P1S) against poliovirus type 1 (PV-1). The cytotoxicity of the compounds was analyzed by MTT and the antiviral effect was determined by plaque reduction assay in different protocols. The polysaccharides did not show any cytotoxic effects on HepG2 cells at the highest tested concentration (200 μg/ml) and exhibited significant antiviral activity with inhibitory concentrations (IC_{50}) of 80 μg/ml, 37.5 μg/ml, 77.5 μg/ml, and 12.1 μg/ml for P1, P1S and the selectivity indexes (SI) ranged from 18 to 131.9. Polysaccharide obtained from *A. indica* act against PV-1 by inhibiting the initial stage of viral replication. Importantly, original polysaccharides showed better virucidal effect than their sulfated derivatives at all tested concentrations.

Badam, Joshi and Bedekar (1999) showed *in vitro* antiviral activity of neem extract against group B coxsackieviruses. Recent research in the defense research and development establishment, Gwalior showed that neem exhibits antiviral activity against dengue virus type-2 replication (Parida et al., 2002). Parida reported *in vitro* and *in vivo* inhibitory potential of crude aqueous extract of neem leaves and pure neem compound (Azadirachtin) on the replication of dengue virus type-2. *In vitro*

antiviral activity of aqueous neem leaves extract assessed in C(6/36) (cloned cells of larvae of *Aedes albopictus*) cells employing virus inhibition assay showed inhibition in dose dependent manner.

1.2.1 (d) Antifungal

Antimicrobial activity of neem oil was documented by Sairam et al. (2000). Efficacy of NIM-76, a spermicidal fraction from neem oil, was investigated for its antimicrobial action against certain bacteria, fungi and polio virus as compared to whole neem oil. The NIM-76 preparation showed stronger anti-microbial activity than the whole neem oil. NIM-76 also exhibited antifungal activity against *Candida albicans* and antiviral activity against polio virus replication in *in vitro* cell lines. It also protected mice from systemic candidiasis as revealed by enhanced percentage of survival and reduced colony forming units of *C. albicans* in various tissues.

1.2.1 (e) Sexually transmitted disease

A. indica activity against sexually transmitted diseases was studied by Kavita and Sanjay (2002). Research is in progress to identify plants and their active principles possessing activity against sexually transmitted pathogens including human immunodeficiency virus (HIV) with an objective of providing an effective approach for prevention of transmission and treatment of these diseases. Plants reported to possess activity or used in traditional systems of medicine for prevention and treatment of STDs including AIDS, herbal formulations for vaginal application, and topical microbicides from herbal origin have been documented. The effect of a neem

herbal formulation in treating abnormal vaginal discharge was studied by Sharma et al. (2009).

Recent research by Shokeen, Bala and Tandon (2009) showed the activity of 16 medicinal plants against *Neisseria gonorrhoeae*. 50% ethanolic extracts of various parts of 16 medicinal plants were evaluated for potential activity against clinical isolates and WHO strains of *N. gonorrhoeae*, including multi drug resistant (MDR) strains. Their activity was calculated as percentage inhibition in comparison with penicillin and ciprofloxacin and the strains were categorised as less sensitive, sensitive or highly sensitive to the extracts. The extracts caused differential inhibition of *N. gonorrhoeae*, with greater inhibition of the MDR strains. Among the extracts tested, 60% exhibited high activity whereas 20% showed moderate activity and 20% had little activity against *N. gonorrhoeae*.

1.2.1 (f) Neem and the immune system

Das et al. (2014) reported the effects on murine carcinoma expressing carcinoembryonic antigen like protein. Researchers generated a polyclonal antibody against a novel immunomodulator, neem leaf glycoprotein (NLGP) that can react to a specific 47 kDa subunit of NLGP. Generated antiNLGP antibody (primarily IgG2a) was tested for its antitumor activity in murine carcinoma (EC, CT-26), sarcoma (S180) and melanoma (B16Mel) tumor models. Tumor growth restriction was only observed in CT-26 carcinoma models, without any alteration in other tumor systems.

Prevention of immune evasion and targeting of STAT3 phosphorylation by neem leaf glycoprotein was studied by Goswami et al. (2014). In this study, researchers

analyzed the *in vitro* immunomodulatory potential of a non-toxic neem leaf glycoprotein (NLGP) in reprogramming stage III M2TAMs induced by supraglottic laryngeal tumor cell lysate (SLTCL) to their classical antitumor shape (M1). Chief defensive macrophages are converted to immunosuppressive M2 type tumor associated macrophages (M2TAMs). M1 macrophages produce various cytotoxic mediators. Inflammatory macrophages in their classical form (M1) detect and destroy invading pathogens and exhibit direct cytotoxicity toward tumor cells *in vitro*. The study postulates a new mechanism of NLGP mediated tumor growth restriction in mice that reflects in better survivability of NLGP treated tumor hosts.

Anti-tumor activity of neem leaf glycoprotein was reported by Mallick et al. (2013). Neem leaf glycoprotein is nontoxic to physiological functions of Swiss mice and Sprague Dawley rats. Neem leaf glycoprotein activates $CD8^+$ T cells to promote therapeutic anti-tumor immunity inhibiting the growth of mouse sarcoma. Collectively, the outcome of the study support a paradigm in which NLGP dynamically orchestrates the activation, expansion, and recruitment of $CD8^+$ T cells into established tumors to operate significant tumor cell lysis.

Immunomodulatory and cancer preventive functions of neem leaf glycoprotein were reported by Baral et al. (2008). The invention provides a process for isolating and characterization of glycoprotein from neem leaf for immunomodulatory and cancer preventive functions of neem leaf glycoprotein (NLGP). The process includes the prepararion of crude neem leaf preparation (NLP) by soaking neem leaf powder in PBS for a period of 24 hours, extensive dialysis of crude NLP thus obtained against PBS, followed by subsequent concentration by membrane filtration, exposing NLGP

to temperature gradient of 0°C to 100°C, exposing the NLGP to different proteolytic enzymes and exposing the NLGP to various ions.

Studies by Chakraborthy et al. (2008) neem leaf glycoprotein restore the impaired chemotactic activity of peripheral blood mononuclear cells from head and neck squamous cell carcinoma patients. Neem leaf glycoprotein is responsible for *in vivo* immunomodulation to restrict the growth of mice tumors. The effect of NLGP in the rectification of the dysregulated IFN gamma-dependent chemokine and its receptor CXCR3 splice variants was investigated.

Chouhan et al. (2015) found that apoptosis mediated leishmanicidal activity of *A. indica* bioactive fractions is accompanied by Th1 immunostimulatory potential. Exploration of immunomodulatory antileishmanials of plant origin is now being strongly recommended to overcome the immune suppression evident during visceral leishmaniasis (VL) and the high cost and toxicity associated with conventional chemotherapeutics. In accordance, researchers assessed the *in vitro* and *in vivo* antileishmanial and immunomodulatory potential of ethanolic fractions of *A. indica* leaves and seeds.

Alti et al. (2015) have also reported the *in vitro* and *in vivo* evaluation of antileishmanial and immunomodulatory activity of neem leaf extract in *Leishmania donovani* infection**.** The study is primarily focused to evaluate the anti-leishmanial effects of neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC to be 52.4µg/ml on the promastigote stage.

Immunomodulatory activity of an aqueous extract of the stem bark of *A. indica* was reported by Van der Nat et al. (1987). The interference of an aqueous extract of the stem bark of *A. indica* with different parts of the human immune system was investigated. The extract showed strong anticomplementary effects which were dose- and time-dependent and most pronounced in the classical complement pathway assay. Moreover, a dose-dependent decrease in the chemiluminescence of polymorphonuclear leukocytes was observed and a dose-dependent increase in the production of migration inhibition factor by lymphocytes.

Immunomodulatory effects of neem (*A. indica*) oil were reported by Upadhyay et al. (1992). The animals were treated intraperitoneally (i.p.) with neem oil, control animals received the emulsifying agent with or without peanut oil. Peritoneal lavage, collected on subsequent days, showed a maximum number of leukocytic cells on day 3 following treatment with neem oil, peritoneal macrophages exhibited enhanced phagocytic activity and expression of MHC class-II antigens.

Immunomodulatory effects of NIM-76, a volatile fraction from neem oil were reported by Sairam et al. (1997). Pre-treatment of rats with a single i.p. injection of NIM-76 resulted in an increase in polymorphonuclear (PMN) leukocytes with a concomitant decrease in lymphocyte counts. The immunomodulatory activity of NIM-76 was found to be concentration-dependent. At 120 mg/kg body weight, there was an enhanced macrophage activity and lymphocyte proliferation response, while the humoral component of immunity was unaffected. The study indicates that NIM-76 acts through cell-mediated mechanisms by activating macrophages and lymphocytes.

Prophylactic as well as therapeutic administration of neem leaf glycoprotein (NLGP) induces significant restriction of solid tumor growth in mice was reported by Banerjee et al. (2014). Researchers investigated the effect of pretreatment of NLGP benefits regulation of tumor angiogenesis, an obligate factor for tumor progression. The study show that NLGP pretreatment results in vascular normalization in melanoma and carcinoma bearing mice along with downregulation of CD31, VEGF and VEGFR2. Accumulated evidences of the study suggested that NLGP regulated immunomodulation is active in tumor growth restriction and normalization of tumor angiogenesis as well, thereby, signifying its clinical translation.

Sarkar et al. (2010) showed that neem leaf glycoprotein enhances carcinoembryonic antigen presentation of dendritic cells to T and B cells for induction of antitumor immunity by allowing generation of immune effector/memory response. Vaccination with neem leaf glycoprotein matured carcinoembryonic antigen (CEA) pulsed dendritic cells (DCs) enhances antigen-specific humoral and cellular immunity against CEA and restricts the growth of CEA(+) murine tumors. NLGP helps better CEA uptake, processing and presentation to T/B cells.

1.2.1 (g) Anticarcinogenic activity

Research (Ratna et al., 2010) showed identification of a cytotoxic activity of neem constituents. Ethanolic neem (*A. indica*) leaf extract induces apoptosis and reported by Perumal et al. (2012). Researchers investigated the molecular mechanisms involved in the induction of apoptosis and the antiproliferative activity exerted by ethanolic neem leaf extract (ENLE) on the human breast cancer cell lines. Two

different breast cancer cell lines such as estrogen dependent (MCF-7) and estrogen independent (MDA-MB-231) cells were exposed to various concentrations of ENLE. Results suggested that ENLE induced apoptosis and decreased cell proliferation through the inhibition of the IGF signalling molecules in both MCF-7 and MDA MB-231 cells, which could be useful for breast cancer treatment.

Antioxidant activity of neem leaves were documented by Manikandan et al. (2008). Researchers evaluated the chemopreventive potential of *A. indica* (neem) leaf fractions based on *in vitro* antioxidant assays, and *in vivo* inhibitory effects on 7,12-dimethylbenzanthracene (DMBA) induced hamster buccal pouch (HBP) carcinogenesis. The results of this study suggest that the antioxidant properties of neem leaf fractions may be responsible for modulating key hallmark capabilities of cancer cells such as cell proliferation, angiogenesis and apoptosis in the HBP carcinogenesis model.

Vinod et al. (2011) reported the mutagenic and antimutagenic activities of neem seed oil. Mutagenic and antimutagenic activity of neem oil (NO) and its DMSO extract were examined in the *Ames Salmonella*/microsome mutagenicity test and the mouse bone marrow micronucleus assay. Eight different strains of *Salmonella typhimurium* were used to study the genotoxicity of neem oil both in the presence and absence of Aroclor-1254 induced rat liver homogenate. Two-dose treatment protocol was employed to study the cytogenetic activity in micronucleus assay. Similarly, the antimutagenic activity of neem oil was studied against mitomycin (MMC) and 7,12-dimethylbenz[a]anthracene (DMBA) in the above two test systems. Neem oil was nonmutagenic in all the eight tester strains of *Salmonella typhimurium*. In the study,

there was no significant increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in neem oil treated groups over the negative control (DMSO) group of animals, indicating the nonclastogenic activity of neem oil in the micronucleus test. Neem oil showed good antimutagenic activity against DMBA induced mutagenicity compared to its DMSO extract. These results indicate nonmutagenic activity of neem oil and significant antimutagenic activity of neem oil suggesting its pharmacological importance for the prevention of cancer.

Trisha et al. (2004) studied the chemopreventive activity of *A. indica* leaf extract.The effect of two different doses (250 and 500 mg per kilogram body weight) of 80% ethanolic extract of the leaves of *A. indica* were examined on drug metabolizing Phase-I and Phase-II enzymes, antioxidant enzymes, glutathione content, lactate dehydrogenase, and lipid peroxidation in the liver of 7-week-old Swiss albino mice. Also anticarcinogenic potential of *A. indica* leaf extract was studied adopting protocol of benzopyrene-induced fore-stomach and 7,12-dimethyl benzanthracene (DMBA) induced skin papillomagenesis. There was a significant inhibition of tumor burden, in both the tumor model system studied (from $P < 0.005$ to $P < 0.001$). Tumor incidence was also reduced by both the doses of *A. indica* extract.

1.2.1 (h) Antifertility

The mechanism of action of neem oil was studied by Sharma et al. (1996). The study was undertaken to elucidate the mechanism of spermicidal action of NIM-76, a fraction isolated from neem oil. The spermicidal activity of NIM-76 was confirmed using a fluorescent staining technique. NIM-76 was found to affect the motility of the sperm in a dose-dependent manner.

Research in the 1990s by Charu and Shakti (1995) showed antifertility activity of neem. The effect of intrauterine neem treatment (IUNT) on ovarian functions and uterine responsiveness to ovarian hormones was examined in adult Wistar rats. Treated animals had normal reproductive cycles as indicated by the vaginal smears; serum progesterone levels were also in the normal range. The study shows that the mode of antifertility action of IUNT is not because of uterine unresponsiveness to the ovarian hormones but due to impairment of embryo development.

Early post implantation contraceptive effects of a purified fraction of neem (*A. indica*) seeds were reported by Mukherjee, Garg and Talwar (1999). The hexane extract of the neem seeds was found to be biologically active and was the precursor for the final active fraction. The active fraction, identified as a mixture of six components, could completely abrogate pregnancy in rodents up to a concentration of 10%. No apparent toxic effects could be seen following treatment with the fraction. The treatment with the active fraction caused a specific activation of T lymphocyte cells of CD8+ subtype as well as phagocytic cells followed by elevation in cytokines gamma-interferon and TNF. The results of the study show that a pure active fraction of neem seeds could be obtained for the purpose of early post implantation contraception when given orally, and its mechanism of action seems to be by activating cell mediated immune reactions.

Anand, Tewari and Mathur (1988) documented the contraceptive action of neem oil in rodents. At subcutaneous doses up to 0.3 ml/rat, neem oil did not possess any estrogenic, antiestrogenic or progestational activity and appeared not to interfere with

the action of progesterone. These findings were confirmed using the histoarchitecture of the uterus of treated rats. Since the postcoital contraceptive effect of neem oil seems to be nonhormonal, neem oil would be expected to elicit fewer side effects than the steroidal contraceptives.

1.2.1 (i) Skin diseases

Neem has a remarkable effect on chronic skin conditions. *A. indica* is active against various skin disorders. Neem oil and leaves have been used for the treatment of skin disorders which was reported by Joseph (2008). Traditional medicines are used to treat various skin disorders such as psoriasis, eczema, alopecia, diabetic ulcer, warts, vitiligo, pemphigus, pompholyx and leprosy.

1.2.1 (j) Antisnake venom activity

Antisnake venom activity and mechanism of action of the active compound isolated from *A. indica* leaves was studied by Ashis et al. (2008). A compound [AIPLAI (*A. indica* PLA(2) inhibitor)] purified from the methanolic leaf extract of *A. indica* inhibits the phospholipase A(2) enzymes in the cobra and Russell's viper venoms (RVVs) in a dose-dependent manner. Inhibition of catalytic and tested pharmacological properties of cobra venom (*Naja naja* and *Naja kaouthia*) PLA(2) enzymes by AIPLAI is significantly higher ($P<0.05$). The study shows that AIPLAI holds good promise for the development of novel antisnake venom drug in future.

1.2.1 (k) Circulatory disorders

Cardioprotective effect of neem leaf extracts was documented by Peer et al. (2008). The study was designed to evaluate the cardioprotective potential of aqueous leaf extract of *A. indica* (AI) on the basis of haemodynamic, biochemical and histopathological parameters in isoprenaline induced myocardial infarction in rats and to compare with vitamin E, a known cardioprotective antioxidant. *A. indica* leaf extract exerts equipotent cardioprotective activity in the experimental model of isoprenalin induced myocardial necrosis in rats as compared to vitamin E, a known cardioprotective antioxidant.

1.2.1 (l) Digestive disorders

Hepatoprotective activity of *A. indica* leaves and bark extract was reported by Devmurari and Jivani (2010). *In vitro* studies involved isolation of hepatocytes and examination of the effect of toxicants along with the test samples. The rat hepatocytes were isolated by recirculating enzymatic perfusion technique (in situ). The lowering of enzyme level is a definite indication of hepatoprotective action of *A. indica*.

Cytoprotective and antisecretory effects of azadiradione were studied by Singh, et al. (2015). Researchers isolated azadiradione from the ethanolic extract of seeds of *A. indica* and evaluated for *in vivo* antiulcer activity in cold restraint induced gastric ulcer model, aspirin induced gastric ulcer model, alcohol induced gastric ulcers model and pyloric ligation induced ulcer model. Azadiradione exhibited potent antiulcer activity through the inhibition of H^+ K^+ATPase (proton pump) activity via its cytoprotective effect and also via its antisecretory effect.

Hepatoprotective activity of azadirachtin-A in carbon tetrachloride intoxicated Wistar rats was studied by Balignar et al. (2014). Hepatoprotective studies revealed that the CCl_4 treatment group exhibited a decrease in total protein and albumin levels, whereas a significant increase in BUN, AST, ALT, and ALP levels were noticed compared with the vehicle treated control, indicating that there was liver damage caused by CCl_4. Histology and ultrastructure study confirmed that pretreatment with azadirachtin-A dose-dependently reduced hepatocellular necrosis and, therefore, protected the liver against toxicity caused by CCl_4.

Antiulcer activity of *A. indica* bark was reported by Uday et al. (2002). The antisecretory and antiulcer effects of aqueous extract of Neem (*A. indica*) bark have been studied along with its mechanism of action, standardisation and safety evaluation. The extract can dose dependently inhibit pylorus ligation and drug induced (mercaptomethylimidazole) acid secretion with ED_{50} value of 2.7 and 2 mg/ kg. respectively. Neem bark extract has therapeutic potential for the control of gastric hyperacidity and ulcer.

Protective role of extracts of neem seeds in diabetes was studied by Gupta (2004). Effect of petroleum ether extracts of kernel (NSK) and husk (NSH) of neem (*Azadirachta indica* A. Juss, Meliaceae) seeds on the prevention of oxidative stress caused by streptozotocin (STZ) was investigated. Diabetes mellitus was induced in adult male Wistar rats after administration of STZ (55 mg/kg b.wt., i.p., tail vein). Results suggest that NSH and NSK prevent oxidative stress caused by STZ in heart and erythrocytes. However, no such preventive effect was observed on renal and hepatic toxicity.

1.2.1 (m) Nervous disorders

The neuroprotective effect of *A. indica* leaf extracts in rats was studied by Sudhirkumar et al. (2005). In the study, bilateral common carotid artery (BCCA) occlusion for 30 min followed by 45 min reperfusion resulted in increase in lipid peroxidation, superoxide dismutase (SOD) activity and fall in total tissue sulfhydryl (T-SH) groups. *A. indica* pretreatment (500 mg/kg/day x 7 days) attenuated the reperfusion induced enhanced lipid peroxidation, SOD activity and prevented fall in T-SH groups. *A. indica* (500 mg/kg/day x 15 days) significantly reduced these hypoperfusion induced functional disturbances. Reactive changes in brain histology like gliosis, perivascular lymphocytic infiltration, recruitment of macrophages and cellular edema following long term hypoperfusion were also attenuated effectively by *A. indica*. The study provides an experimental evidence for possible neuroprotective potentiality of *A. indica*.

1.2.1 (n) Parasitic diseases

Inhibition of the growth and development of asexual and sexual stages of drug sensitive and resistant strains of the human malaria parasite *Plasmodium falciparum* by neem (*A. indica*) fractions was reported by Ravi et al. (1998). In the study researchers systematically evaluated extracts of neem seeds and purified fractions further enriched in polar or non-polar constituents for their effect on *in vitro* growth and development of asexual and sexual stages of the human malaria parasite *Plasmodium falciparum*.

Lucantoni et al. (2006) showed the effects of a neem extract on blood feeding, oviposition and oocyte in *Anopheles stephensi.* In this study, a laboratory strain of *Anopheles stephensi* was used to assess the effects of a commercial formulation (Neem Azal), containing azadirachtin A at 34%, on blood feeding, oviposition and oocyte ultrastructure. Oral administration of neem azal to *A. stephensi* females through artificial blood meals did impair blood intake and oviposition in a concentration dependent manner. The study indicates that neem impairs hormone control of oogenesis and exerts a cytotoxic effect on both follicular cells and oocytes of the Asian malaria vector *A. stephensi*.

1.2.2 Neem in animal health

A. indica has been used in the studies of the treatment of a large number of diseases in animals ranging from systemic disorders to infections and was reported by Sudipta et al. (2010). In this study, researchers analyzed the pectic arabinogalactan isolated from *A. indica* and its chemically sulfated derivative. These macromolecules showed activity against bovine herpesvirus type-1. Their inhibitory concentration 50% values ranging from 31.12 to 105.25 μg/ml were lower than the cytotoxicity values (>1600–1440 μg/ml). The anti-viral effect was exerted during virus adsorption to the cell. Anionic groups in particular the sulfate groups appeared to be very important for the anti-herpetic activity of these polymers.

The *A. indica* bark is reported to be beneficial in cutaneous diseases. Antibacterial agent against fish bacteria was studied by Das et al. (1999). Aquaneem, an emulsified product prepared from the neem (*A. indica*) kernel was tested against four pathogenic bacteria of fish (*Aeromonas hydrophila, Pseudomonas fluorescens, Escherichia coli*

and *Myxobacteria spp*.) to test its efficacy as an antibacterial agent. Growth inhibitory property of the product at 10, 15 and 20 ppm has been noticed and recorded. Among all the bacteria tested *A. hydrophila*, *P. fluorescens* and *Myxobacteria spp*. exhibited maximum sensitivity to Aquaneem in terms of percentage reduction of bacterial cell population in comparison to *E. coli*. Yunxia et al. (2012) shows the acaricidal activity of petroleum ether extract of *A. indica* oil against *Sarcoptes scabiei* var. *cuniculi in vitro*. The acaricidal bioassay was conducted using a dipping method. The results indicated that the median lethal concentration (LC50) of the petroleum ether extract (at the concentration of 500.0ml/l) was 70.9ml/l, 24h after treatment. The results suggest that petroleum ether extracts of neem oil and its four fractions possess *in vitro* acaricidal activity.

1.3 Neem tree

1.3.1 Parts of neem

The neem tree grows upto 30 m tall and has a girth of 2.5 m. It has straight trunk, which has a diameter of 30-80 cm (Mohammad, 2016). The branches of the neem tree spread across 20m. The leaves, bark, root and seeds of the neem trees have numerous medicinal properties for the treatment of many diseases. The leaves of the neem are bitter in taste. They are medium to dark in colour and 3-8 cm long. The flowers of the neem tree are white in colour. The inflorescence bear from 150-250 flowers and each flower is 5-6 mm long and 10 mm wide. The neem fruit is oval or round in shape. The skin of the fruit is very thin and called endocarp. Mesocarp is the pulp which if fibrous and bitter sweet to taste. The fruits are yellowish white in colour. The endocarp is the neem seed which is white in colour with a brown coating. The inner shell is very hard.

1.3.2 Chemical Constituents

The neem compounds have been divided into two major categories such as isoprenoids and nonisoprenoids (Figure 1.2 – 1.22). The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and csecomeliacins such as nimbin, salanin and azadirachtin (Morgan, 2009). Tetracyclic triterpenoids was isolated from the leaves of *A. indica* by Siddiqui et al. (2004). The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids, flavonol glycosides, dihydrochalcone, coumarin, tannins and aliphatic compounds.

1.3.2 (a) Isoprenoids – diterpenoids

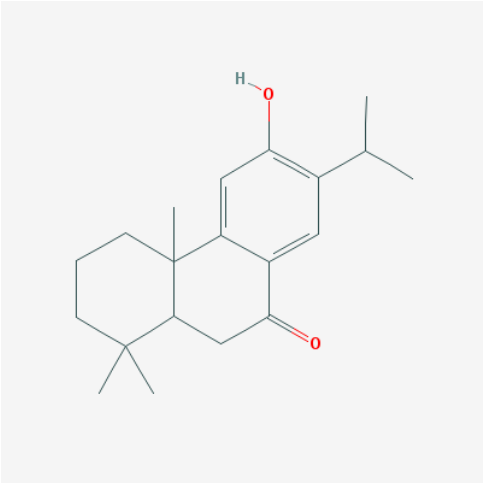

Figure 1.2 Sugiol

Molecular Formula: $C_{20}H_{28}O_2$

Molecular Weight : 300.442