4 *Azadirachta indica* (Neem) and Neem Limonoids as Anticancer Agents: Molecular Mechanisms and Targets

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Abstract

Neem (*Azadirachta indica* A. Juss), one of the most versatile medicinal plants that grows ubiquitously in India has attained worldwide prominence owing to its wide range of medicinal properties. The bioactivity of neem has been attributed to its rich content of complex limonoids. Neem extracts and limonoids have been documented to exert antiproliferative effects both in vitro and in vivo. Accumulating evidence indicates that the anticancer effects of neem extracts and neem limonoids are mediated by preventing carcinogen activation; enhancing host antioxidant and detoxification systems; inhibiting cell proliferation, inflammation, invasion and angiogenesis; inducing apoptosis; modulating oncogenic transcription factors and signalling kinases; and influencing the epigenome. Neem and its constituent limonoids that target multiple signalling pathways aberrant in cancer are promising candidates for anticancer drug development.

Keywords

Anticancer • Azadirachtin • Nimbolide • Limonoids • Neem

Abbreviations

Apaf-1	Apoptosis associated factor-1
Bcl-2	B-cell lymphoma-2
B(a)P	Benzo(a)pyrene
CDK	Cyclin-dependent kinases
DR	Death receptor
Dvl	Dishevelled
DMBA	7,12-dimethylbenz(a)anthracene
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
GSK-3β	Glycogen synthase kinase 3 beta
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1α
IAPs	Inhibitors of apoptosis proteins
IκB-α	Inhibitor of kappa B alpha
IKK	IκB kinase
IL	Interleukin
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases

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MTMP	Mitochondrial permeability transition pore
NF-κB	Nuclear factor kappa B
NLGP	Neem leaf glycoproteins
PCNA	Proliferating cell nuclear antigen
PARP	Poly(ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
ROS	Reactive oxygen species
Smac/Diablo	Second mitochondria derived activator of caspases/direct IAP binding protein with low pI
TIMP-2	Tissue inhibitor of matrix metalloproteinases-2
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Ub	Ubiquitin ligase
VEGF	Vascular endothelial growth factor
XME	Xenobiotic metabolizing enzyme

4.1 Introduction

Azadirachta indica A. Juss (*Meliaceae*), commonly known as neem, is a large, evergreen tree belonging to the *Meliaceae* family ubiquitously present in the Indian subcontinent. Neem is one of the most researched trees globally because of its beneficial effects on the ecosystem and health. Neem is used both as a fertilizer and as a pesticide. The neem tree is recognized to control soil erosion and salinity and improve fertility of the soil. Biopesticides manufactured from neem are effective, eco-friendly, and acceptable to the farmers. Neem products have been shown to possess insecticidal, larvicidal, and mosquito-repellent properties (van der Nat et al. 1991; Udeinya 1993; Brahmachari 2004). Neem seeds are the richest source of pesticidal and insecticidal compounds. In particular, azadirachtin, the most active principle of neem-based insecticides and pesticides extracted from neem seed kernels, has been documented to be effective against approximately 200 insect pests (Mordue and Blackwell 1993; Morgan 2009).

The neem tree has gained worldwide attention in recent years, owing to its wide spectrum of medicinal properties. All parts of the neem tree—leaves, flowers, seeds, fruits, roots, and bark—have been used in traditional systems of medicine including Ayurveda, Siddha, Unani, Roman, and Greek to treat numerous human ailments. In India, the neem tree is considered as '*sarva roga nivarini*' (the panacea for all diseases) and has been hailed as '*heal all*', '*divine tree*', '*village dispensary*', and '*nature's drug store*' (Puri 1999).

The neem is a broad-leaved tree that can grow up to 30 m in height with branches spreading over 20 m and roots that deeply penetrate the soil. The trunk has a moderately thick, furrowed bark. The leaves are broad, alternate, and bitter in taste, and the flowers and fruits are borne in axillary clusters. The drupes are ellipsoidal and greenish yellow in colour and comprise a sweet pulp enclosing the seed composed of shell and kernels. Neem seed oil accounting for about 45 % of the total weight of the seeds is extracted from the kernel that contains secretory cells, which are the sites for the synthesis and storage of neem chemicals (Puri 1999; Biswas et al. 2002; Brahmachari 2004).

4.1.1 Chemistry

Over 300 structurally complex bioactive, organic compounds have been isolated and characterized from various parts of the neem tree (Kumar et al. 1996; Biswas et al. 2002; Tan and Luo 2011). These compounds have been categorized into two major classes: isoprenoids or terpenoids and non-isoprenoids. The isoprenoids include diterpenoids, triterpenoids, vilasinin type of compounds, and C-secomeliacins. Among these, the triterpenoids are the most abundant and occur in all parts of the neem tree especially in seeds and leaves. These are categorized into protolimonoids and mono- to nonanortriterpenoids. The tetranortriterpenoids are also known as limonoids. The non-isoprenoids include polysaccharides, proteins, amino acids, sulphur compounds, hydrocarbons, fatty acids, and their esters, tannins, and

polyphenolics such as flavonoids and coumarin (Biswas et al. 2002; Subapriya and Nagini 2005).

Although a large number of compounds have been isolated from neem, only a few pure compounds have been screened for biological activity as shown in Table 4.1. The bioactivity of neem has been largely attributed to the rich content of complex limonoids that constitute about one-third of the phytochemical constituents in neem. Limonoids are highly oxygenated modified triterpenes categorized as tetranorterpenoid. Limonoids with an intact apoeuphol skeleton, a 14,15-β-epoxide, and a reactive site (either 19–28 lactol bridge or a cyclohexane) on the A ring are biologically very active, and absence of these structural features results in reduced activity (Tan and Luo 2011). Activity-guided fractionation of crude ethanolic extract of neem leaf revealed the presence of nimbolide, nimbin, 2′3′-dehydrosalannol, 6-desacetyl nimbinene, nimolinone, and quercetin (Manikandan et al. 2008, 2009; Mahapatra et al. 2012).

4.1.2 Medicinal Properties

Of late, neem has attracted increasing research attention because of the plethora of health benefits that it confers. Although all parts of the neem tree have been successfully used for centuries to treat a variety of disorders, the medicinal utilities have been described especially for leaf and seeds (Biswas et al. 2002; Brahmachari 2004; Subapriya and Nagini 2005). Extracts of neem seeds and leaves were shown to provide protection against various strains of malarial parasite and human fungi including *Candida* (Badam et al. 1987; Khan and Wassilew 1987). The antibacterial effects of neem leaves, seeds, and bark have been demonstrated against Gram-positive and Gram-negative bacteria including *M. tuberculosis* and *Streptomycin* resistant strains, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *M. pyogenes* (Chopra et al. 1952; Satyavati et al. 1976). Neem and neem-based products exhibit antiviral activity against chikungunya, herpes simplex virus-1, dengue virus type-2, and human immunodeficiency virus (HIV) (Udeinya et al. 2004).

Alcoholic neem leaf extracts were found to be effective against chronic skin diseases such as eczema, scabies, and ringworm infection (Singh et al. 1979). Neem has been used in the treatment of gingivitis, periodontitis, oral infections, and inhibition of plaque growth (Patel and Venkatakrishna-Bhatt 1988). A dental gel containing neem leaf extract (25 mg/g) was documented to reduce plaque index and bacterial count (Pai et al. 2004). Herbal chewing sticks of neem were found to be effective against *Streptococcus* spp. involved in causing dental caries (Prashant et al. 2007). Both the aqueous and alcoholic neem leaf extracts are reported to exhibit hepatoprotective effects against liver damage induced by paracetamol and antitubercular drugs (Bhanwra et al. 2000; Chattopadhyay 2003). Neem preparations were demonstrated to be useful in the control of gastric hyperacidity and ulcer by blocking acid secretion through inhibition of H^+–K^+-ATPase and by prevention of oxidative damage and apoptosis (Chattopadhyay et al. 2004). Studies have documented that oral administration of aqueous as well as alcoholic extract of neem leaf decreased blood glucose level in experimentally induced diabetes by releasing endogenous insulin (Khosla et al. 2000).

Neem extracts have been shown to exert potent anticancer effects attributed to the presence of limonoids (Roy and Saraf 2006). Azadirachtin isolated from seed kernels, and nimbolide, abundant in neem leaves and flowers are the most active neem limonoids that display anticancer effects.

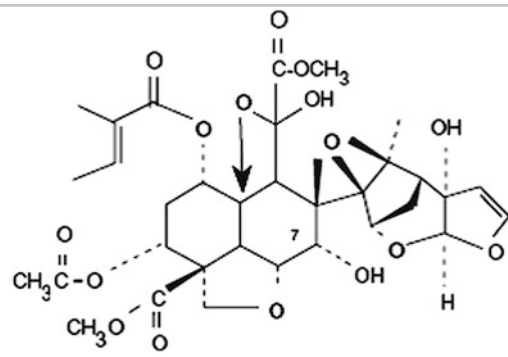
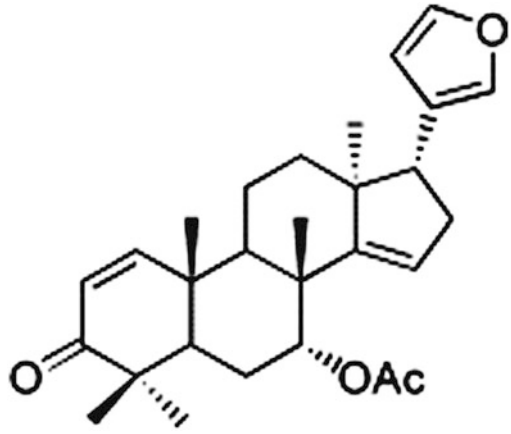
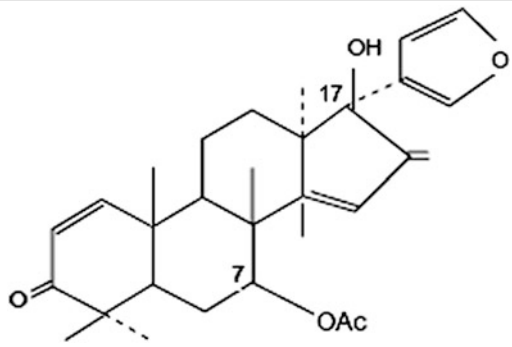
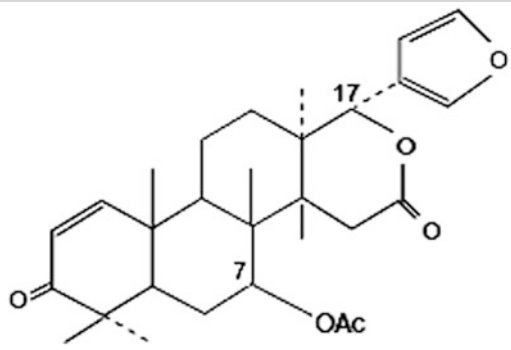
4.2 Anticancer Properties

The evidence for the anticancer property of neem extracts and neem limonoids stems from both in vitro and in vivo studies (Biswas et al. 2002; Subapriya and Nagini 2005; Paul et al. 2011).

4.2.1 In Vitro Studies

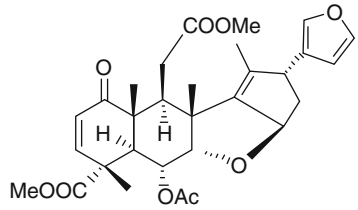
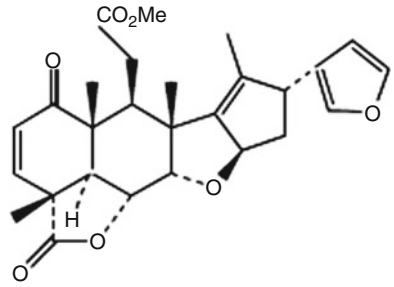
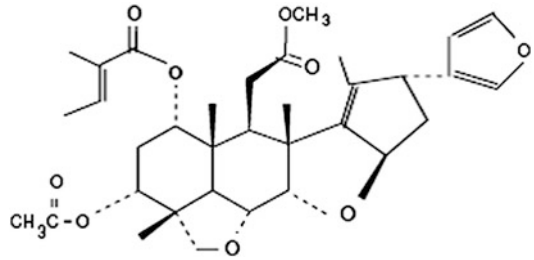
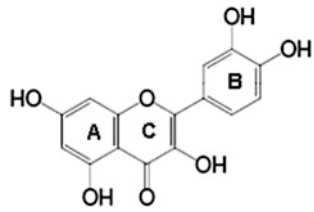
Several studies have demonstrated the inhibitory effects of neem extracts and limonoids on the

Table 4.1 Structure and biological activity of neem phytochemicals

Neem compound	Structure	Biological activity
Azadirachtin		Insecticidal, antimicrobial, antioxidant, antimutagenic, anticancer
Azadirone		Anticancer
Deacetyl nimbin		Spermicidal, anticancer
Gedunin		Antimicrobial, diuretic, antiarthritic, antipyretic, spermicidal, anti-inflammatory, anti-ulcerogenic, anticancer

(continued)

Table 4.1 (continued)

Neem compound	Structure	Biological activity
Nimbin		Spermicidal, anticancer
Nimbolide		Insecticidal, antimicrobial, cytotoxicity, antioxidant, antimutagenic, anticancer
Salannin		Antigastric ulcer, anticancer
Quercetin		Antioxidant, anticancer

growth of diverse cancer cells in vitro. Neem leaf extracts exerted anticancer effects against human pancreatic carcinoma cell lines Panc-1, BxPC-3, and MIA PaCa-2; prostate cancer cells such as LNCaP, C4-2B, and PC-3; as well as murine Ehrlich's carcinoma (EC) and B16 melanoma cells (Baral and Chattopadhyay 2004; Gunadharini et al. 2011; Mahapatra et al. 2011; Veeraraghavan et al. 2011a, b).

Among the 35 limonoids from *Azadirachta indica* seed extracts evaluated for cytotoxicity against five human cancer cell lines, three limonoids 7-deacetyl-7-benzoylepoxyazadiradione, 7-deacetyl-7-benzoylgeduin, and 28-deoxonimbolide displayed potent cytotoxic effects against HL60 leukaemia cells with IC_{50} values in the range 2.7–3.1 μM. In particular, 7-deacetyl-7-benzoylepoxyazadiradione exhibited selective cytotoxicity to leukaemic cells and only weak cytotoxicity against the normal RPMI 1788 lymphocyte cell line (Kikuchi et al. 2011).

Azadirachtin has been reported to have potent cytotoxic effects against glioblastoma cell lines (G-28, G-112, G-60, G-44, G-62, G-120) by increasing micronuclei formation and decreasing the mitotic index (Akudugu et al. 2011). Azadirachtin was also demonstrated to inhibit the growth of human cervical cancer cells (HeLa),

MCF7 breast cancer cells, 143B.TK$^-$ human osteosarcoma, and ovarian cell lines (Cohen et al. 1996a; Priyadarsini et al. 2010). Nanduri et al. (2003) have documented the cytotoxic effect of azadirone against a panel of cancer cell lines.

Gedunin was shown to manifest anticancer activity via inhibition of the 90 kDa heat shock protein 90 (hsp90) folding machinery in MCF-7 and SkBr3 breast cancer cells (Brandt et al. 2008). Kamath et al. (2009) found that gedunin inhibits the proliferation of SKOV3, OVCAR4, and OVCAR8 ovarian cancer cell lines and enhances the antiproliferative effect of cisplatin. 2′-3′-Dehydrosalannol has been reported to inhibit the growth of MDA-MB 231 and MDA-MB 468 triple-negative breast cancer cells (Boopalan et al. 2012).

Studies have revealed that nimbolide is the most potent anticancer agent among the various neem limonoids examined. Nimbolide has been documented to exert significant cytotoxic effects against a panel of human cancer cell lines including 143B TK osteosarcoma, HL-60, U-937 and THP-1 leukaemic, B16 melanoma, SMMC 7721, A-549, MCF-7 breast, HT-29, SW-620, SW-480, HOP-62, A-549, PC-3, and OVCAR-5 cell lines (Cohen et al. 1996b; Sastry et al. 2006; Roy et al. 2007; Chen et al. 2011). Studies from this laboratory as well as by others have shown that nimbolide induces a dose- and time-dependent suppression of the viability of human choriocarcinoma (BeWo), leukaemic (HeLa), and WiDr and HCT-116 colon adenocarcinoma cells (Harish Kumar et al. 2009; Priyadarsini et al. 2010; Babykutty et al. 2012).

4.2.2 In Vivo Studies

Accumulating evidence from experimental animal models have provided convincing evidence to indicate that neem preparations and its constituents are highly effective in affording protection against malignant tumours induced by a variety of chemical carcinogens. Tepsuwan et al. (2002) demonstrated the chemopreventive potential of neem flowers on carcinogen-induced rat mammary and liver carcinogenesis. Extensive investigations from this laboratory provide evidence that neem leaf extracts inhibit the development of experimental oral and gastric carcinogenesis (Subapriya and Nagini 2003; Subapriya et al. 2005, 2006). Dasgupta et al. (2004) reported the chemopreventive potential of neem leaf extract in murine carcinogenesis model systems. Gangar et al. (2006) have demonstrated that *A. indica* exerts chemopreventive effects against benzo(a)pyrene-induced forestomach murine tumours. Oral administration of aqueous neem leaf extract was demonstrated to significantly reduce the incidence of N-nitrosodiethylamine (NDEA)-induced hepatocellular carcinomas and delayed changes in hepatocyte differentiation, metabolism, and morphology (Bharati and Rishi 2012). Neem leaf was found to exhibit short-term chemopreventive effects on preneoplastic lesions in rat colon carcinogenesis (Arakaki et al. 2006). Haque and Baral (2006) have shown that neem leaf preparation induces Ehrlich carcinoma prophylactic growth restriction in Swiss and C57BL mice. Recently, Mahapatra et al. (2011) have demonstrated that ethanolic neem leaf extracts inhibit the growth of C4-2B and PC-3M-luc2 prostate cancer xenografts in nude mice.

Although neem limonoids have been extensively tested for cytotoxicity against a panel of human cancer cell lines, evidence for the in vivo inhibition of tumour growth in animal models is rather scanty. Limonin 17-β-D-glucopyranoside, a neem limonoid, was shown to inhibit DMBA-induced oral carcinogenesis (Miller et al. 1992). Nortriterpenoids, isolated from the seed extract of neem, exhibited marked inhibitory effect on melanogenesis in the B16 melanoma cells. Akihisa et al. (2009) have documented the anti-tumour-initiating activity of azadirachtin B isolated from the seed extract of neem on the two-stage carcinogenesis of mouse skin tumour induced by peroxynitrite (ONOO−; PN) as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter. Recently, we have demonstrated that administration of both azadirachtin and nimbolide inhibit the development of 7,12-dimethylbenz(a)anthracene

(DMBA)-induced hamster buccal pouch carcinomas by modulating the hallmark capabilities of cancer (Vidya Priyadarsini et al. 2009; Harish Kumar et al. 2010).

4.3 Molecular Mechanisms Underlying Anticancer Effects

4.3.1 Carcinogen Metabolism

A key molecular mechanism responsible for the anticancer effects of neem and its limonoids is the modulation of phase I and II xenobiotic metabolizing enzymes (XME), which play a central role in xenobiotic/drug metabolism. Phase I enzymes, namely, cytochrome P450 monooxygenases and their isoforms, catalyze the biotransformation of procarcinogens to highly reactive electrophilic intermediates that can damage cellular macromolecules. In contrast, the phase II enzymes such as glutathione *S*-transferases (GSTs), uridine diphosphate-glucuronosyl transferases (UGTs), and NADH quinine oxidoreductase (NQO1) catalyze the neutralization of electrophilic intermediates generated in phase I reactions, thereby resulting in reduced chemical reactivity and cellular damage (Iyanagi 2007).

Ethanolic neem leaf extracts, neem leaf fractions, and the limonoids azadirachtin and nimbolide function as *dual-acting agents* offering protection against chemically induced carcinogenesis by constraining the activities of total cytochrome P450 as well as its isoforms CYP1A1, 1A2, 2B and CYP1B1, and cytochrome b_5 and simultaneously enhancing the activities of the phase II detoxification enzymes—GST and DT-diaphorase (Manikandan et al. 2008, 2009; Vidya Priyadarsini et al. 2009). Aqueous extracts of neem leaf were shown to inhibit the formation of benzo(a)pyrene (B(a)P) DNA adducts during B[a]P-induced murine forestomach carcinogenesis via modulating the activities of phase I and II XMEs (Gangar et al. 2006).

4.3.2 Antioxidants

Extensive investigations have provided evidence for the antioxidative properties of neem leaves, fruits, flowers, and stem bark extracts as well as the neem limonoids azadirachtin and nimbolide against various free radicals both in vitro and in vivo (Hanasaki et al. 1994; Sithisarn et al. 2005, 2007; Manikandan et al. 2008, 2009; Vidya Priyadarsini et al. 2009). Farah et al. (2006) reported that ethanolic neem leaf extract exerts significant antimutagenic activity against pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid-induced chromosomal aberrations and incidence of micronuclei in *Channa punctatus*. Studies from this laboratory have unequivocally demonstrated that ethanolic neem leaf extract significantly mitigates carcinogen-induced genotoxicity and oxidative stress by augmenting GSH-dependent antioxidant defence mechanisms (Subapriya et al. 2004, 2005). Activity-guided fractionation identified subfractions that displayed significant protective effects against various free radicals and H_2O_2-induced oxidative damage to erythrocytes and pBR322 DNA (Manikandan et al. 2009). Both azadirachtin and nimbolide exhibited concentration-dependent ROS scavenging activity and reductive potential in vitro and protected against oxidative DNA damage in vivo by upregulation of antioxidants (Vidya Priyadarsini et al. 2009).

4.3.2.1 Cell Proliferation, Cell Cycle Arrest, and DNA Repair

Perturbation of cell cycle control with consequent uncontrolled cell proliferation is a major hallmark of cancer. Sequential activation and deactivation of cyclins and cyclin-dependent kinases (CDKs) regulates progression of the cell cycle through the various phases (Csikasz-Nagy et al. 2011).

Ethanolic extracts of neem leaf have also been reported to inhibit cell proliferation and tumour growth by downregulating a wide array of proteins involved in cellular assembly and

organization, DNA replication, recombination, and repair such as HMOX1, AKR1C2, AKR1C3, and AKR1B10 (Mahapatra et al. 2011). Niture et al. (2006) showed that ethanolic and aqueous extracts of neem reduce the expression of O6-alkylguanine lesions in human peripheral blood lymphocytes by increasing the levels of O6-methylguanine-DNA methyltransferase (MGMT) repair protein. Neem limonoids have been documented to induce cell cycle arrest at G1/S or G2/M phase accompanied by p53-dependent accumulation of $p21^{Cip1/waf1}$ and Chk2 associated with downregulation of the cell cycle regulatory proteins cyclin A, cyclin B1, cyclin D1, cyclin E, Cdk2, Rad17, PCNA, and c-myc (Roy et al. 2006; Priyadarsini et al. 2010). 2′3′-Dehydrosalannol has been shown to inhibit the growth of triple-negative breast cancer cells by downregulating phosphorylated protein kinase B (pAKT) and cyclin D1 (Boopalan et al. 2012). Azadirachtin exerts antimitotic effects by interfering with the polymerization of tubules and formation of the mitotic spindle, thereby preventing replication (Salehzadeh et al. 2003). Studies in vitro and in vivo have revealed the regulatory effects of nimbolide on cell cycle progression. Treatment of U937 cells with nimbolide disrupted the cell cycle by decreasing the number of cells in G0/G1 phase (Roy et al. 2007). In colon cancer cells, nimbolide was shown to interfere with cell cycle kinetics and induce S phase arrest by inhibiting cyclin A/cyclin D1 (Babykutty et al. 2012).

4.3.2.2 Induction of Apoptosis

Apoptosis, a form of programmed cell death, plays a crucial role in the maintenance of adult tissue homeostasis. Apoptosis is initiated via complex interactions between the pro- and anti-apoptotic members of the Bc-2 family that dictate the integrity of the mitochondrial membrane. Permeabilization of the outer mitochondrial membrane by pro-apoptotic Bcl-2 family proteins results in the efflux of apoptogenic factors such as cytochrome c, SMAC/DIABLO, and Omi/HtrA2 from the mitochondrial intermembrane space into the cytosol. In the cytosol, cytochrome c engages apoptotic protease activating factor-1 (APAF-1) and pro-caspase-9 to form the apoptosome complex. Formation of the apoptosome complex subsequently activates the downstream caspase cascade and cleavage of vital proteins essential for cell survival (Ulukaya et al. 2011).

Neem leaf extracts induce apoptosis in human neuroblastoma xenografts, Panc-1, BxPC-3, MIA PaCa-2, LNCaP, and PC-3 cancer cells by modulating the expression of Bcl-2 family proteins and upregulating caspases (Gunadharini et al. 2011; Kumar et al. 2006; Baral and Chattopadhyay 2004; Mahapatra et al. 2011; Veeraraghavan et al. 2011a, b).

Neem limonoids have been shown to induce apoptosis in various cancer cells as evidenced by detachment of cells from the substratum, an increase in the number of sub-diploid cells, chromatin condensation, and appearance of annexin-V-positive cells. Studies by us and other workers have revealed that neem limonoids transduce apoptosis by both the death receptor and mitochondrial pathways (Harish Kumar et al. 2009; Priyadarsini et al. 2010). Gupta et al. (2011) reported that nimbolide sensitizes colon cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by enhancing the expression of the death receptors DR5 and DR4 through activation of ERK and p38 MAP kinase and generation of ROS. Recently, Babykutty et al. (2012) also demonstrated that nimbolide induces caspase-mediated apoptosis by inhibiting ERK1/2 and activating p38 and JNK1/2.

Accumulating evidence indicates that neem limonoids induce apoptosis via the mitochondrial pathway. The mechanism involved generation of reactive oxygen species (ROS); decline in the mitochondrial transmembrane potential; downregulation of anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-xL; upregulation of pro-apoptotic Bax, Bak, Bim, Bid, and p53; reduced expression of inhibitor of apoptosis proteins (IAPs), namely, survivin, neuronal apoptosis inhibitory protein (NAIP), IAP-1, IAP-2, I-FLICE, and XIAP; release of cytochrome *c* from the mitochondria; apoptosome complex formation; and

activation of initiator and effector caspases and CARD domains, eventually culminating in poly (ADP-ribose) polymerase (PARP) cleavage. Interestingly, azadirachtin and nimbolide enforced nuclear localization of survivin, enabling increased susceptibility to intrinsic apoptosis (Gupta et al. 2010, 2011; Priyadarsini et al. 2010; Boopalan et al. 2012).

4.3.2.3 Inhibition of Tumour Invasion and Angiogenesis

Cancer cell invasion and endothelial transmigration, critical events in tumour progression and metastasis, depend on an intricate balance between proinvasive and proangiogenic factors and their inhibitors. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play a pivotal role in extracellular matrix (ECM) processing during which process several proangiogenic molecules predominantly vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) are released. MMP activity is tightly regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (Brew and Nagase 2010; Gialeli et al. 2011; Nagini 2012).

Ethanolic neem leaf extracts are reported to exhibit antiangiogenic effects in human umbilical vein endothelial cells (HUVECs) by attenuating VEGF stimulation (Mahapatra et al. 2012). Studies from this laboratory have provided evidence that the neem limonoids azadirachtin and nimbolide induce a shift of balance from a proinvasive, proangiogenic phenotype by downregulating the expression of MMPs, VEGF, VEGF receptors, and HIF-1α and upregulating TIMP-2 and RECK expression in the hamster buccal pouch carcinogenesis model (Vidya Priyadarsini et al. 2009). In a recent study, nimbolide was demonstrated to block tumour cell invasion, migration, and angiogenesis in colon cancer cells in vitro by downregulating the expression of MMP and VEGF via abrogation of ERK1/2 and NF-κB signalling (Babykutty et al. 2012).

4.3.2.4 Modulation of Oncogenic Transcription Factors

Neem extracts and its constituent limonoids are reported to modulate various transcription factors associated with oncogenesis, chiefly nuclear factor kappa B (NF-κB) and β-catenin. In unstimulated cells, NF-κB exists in the cytosol as an inactive heterodimer of p50 and p65 subunits tightly sequestered to the IκB inhibitory protein. Activation of NF-κB occurs through phosphorylation of IκB at serine-32 and serine-36 residues by IKKβ. This results in proteasomal degradation of IκB and subsequent translocation of free NF-κB heterodimer to the nucleus. In the nucleus, NF-κB binds to the κB enhancer element and transactivates over 500 target genes that are implicated in various processes such as cell proliferation, cell survival, apoptosis evasion, invasion, metastasis, and angiogenesis (Chaturvedi et al. 2011).

Neem extracts and limonoids restrict NF-κB signalling by inhibiting the kinase activity of IKKβ, phosphorylation and proteasomal degradation of IκB, and nuclear translocation of the p50/p65 heterodimer (Manikandan et al. 2008; Priyadarsini et al. 2010; Kavitha et al. 2012). Veeraraghavan et al. (2011a) showed that neem leaf extracts inhibit proliferation of irradiated Panc-1, BxPC-3, and MIA PaCa-2 pancreatic cancer cells through selective abrogation of radiotherapy-induced NF-κB signalling. Gupta et al. (2010) demonstrated that nimbolide inhibits NF-κB activation by modifying cys179 residue in the activation loop of IKKβ, thereby abrogating its kinase activity and subsequent NF-κB signalling. Recent studies from our laboratory have demonstrated that nimbolide inhibits both the constitutive as well as tumour necrosis factor-α (TNF-α)-induced NF-κB activation in NF-κB-responsive luciferase reporter plasmid transfected human hepatocarcinoma (HepG2) cells.

Reciprocal activation of NF-κB and Wnt/β-catenin signalling pathways has been documented in malignant tumours (Du and Geller 2010). Activation of the Wnt/β-catenin pathway involves dissociation of a multiprotein complex containing glycogen synthase kinase 3β

(GSK-3β) that results in cytosolic accumulation and nuclear translocation of β-catenin that interacts with TCF/lymphoid enhancer factor (LEF) to form a functional transcription factor to transactivate various target genes involved in cell proliferation, apoptosis, invasion, and angiogenesis (Sethi and Vidal-Puig 2010). Treatment of HepG2 cells with nimbolide abrogated canonical Wnt/β-catenin signalling by downregulating GSK-3β and impeding the cytosolic accumulation and nuclear translocation of free β-catenin (Kavitha et al. 2012).

4.3.2.5 Modulation of Intracellular Signalling Cascades

The upstream components of the cytoplasmic signalling networks include the protein kinase B/Akt, mitogen-activated protein kinases (MAPKs), protein kinase C, and phosphatidylinositol-3-kinase (PI3K). Inappropriate regulation of these kinases transmit mitogenic signals to transcription factors, co-activators, and co-repressors resulting in transcription of target genes that promote carcinogenesis. Akt, a serine/threonine kinase, plays a pivotal role in various cellular processes particularly cell proliferation and survival. The Akt cascade is activated by receptor tyrosine kinases, integrins, and various other stimuli through the production of phosphatidylinositol 3,4,5 triphosphates. Akt influences cell growth and proliferation through its effects on the mTOR and p70 S6 kinase pathways, cyclin D1, p53, and the CDK inhibitors- p21 and p27. Akt also mediates cell survival through direct inhibition of pro-apoptotic molecules such as Bad and the Forkhead family of transcription factors (Jazirehi et al. 2012). Mitogen-activated protein kinases (MAPK) comprising extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinases are conserved signalling modules that play a key role in oncogenic transformation. Upon activation, these kinases integrate various extracellular and intracellular cues, translocate to the nucleus and induce transcriptional programmes involved in cell growth, apoptosis evasion and matrix invasion (Cargnello and Roux 2011).

Neem extracts and limonoids have been shown to target Akt, phosphoinositide 3-kinase (PI3K), p38, JNK1/2, and ERK signalling pathways and promote apoptosis induction and NF-κB abrogation in diverse malignant cell lines. Gunadharini et al. (2011) reported that ethanolic neem leaf extracts decreased the expression of Akt 1/2 as well as the expression of pAkt and total Akt in PC-3 and LNCaP prostate cancer cells. Babykutty et al. (2012) demonstrated that nimbolide modulates the expression of pERK1/2, pP38, and pJNK1/2 in WiDr colon adenocarcinoma cells. Gupta et al. (2010) found that nimbolide targets ERK and p38 MAPK molecules via generation of reactive oxygen species (ROS) in HCT-116 colon adenocarcinoma cells.

4.3.2.6 Anti-inflammatory Effects

Neem leaf extracts and limonoids have been reported to exert potent anti-inflammatory effects by inhibiting the activation of tumour necrosis factor (TNF)-α, a multifunctional pro-inflammatory cytokine that plays a key role in inflammation through signalling via TNFR1 and TNFR2 and by inhibiting NF-κB activation. Azadirachtin has been shown to exert anti-inflammatory effects via modulating cell surface TNF receptors, thereby blocking TNF-induced inflammatory responses. Inhibition of TNF activation by azadirachtin reduces NF-κB activation and the expression of NF-κB dependent pro-inflammatory mediator COX-2 (Thoh et al. 2010). Epoxyazadiradione is demonstrated to inhibit macrophage migration inhibitory factor (MIF)-mediated pro-inflammation activities in RAW 264.7 cells by inhibiting MIF-induced macrophage chemotactic migration, NF-κB nuclear translocation, and upregulation of inducible nitric oxide synthase and nitric oxide production. Epoxyazadiradione also exhibits anti-inflammatory activity in BALB/c mice by preventing the release of pro-inflammatory cytokines TNF-α and interleukin (IL)-1α, IL-1β, and IL-6 (Alam et al. 2012). The limonoids 1,3-diacetylvilasinin, 28-deoxonimbolide, salannin, 2′,3′-dihydrosalannin, and 3-deacetylsalannin were also found to

exhibit potent anti-inflammatory activity against TPA-induced inflammation (Akihisa et al. 2011).

4.3.2.7 Immunomodulatory Effects

Immunosuppression occurring as a result of increased cytokine secretion is a key phenomenon that promotes tumour growth and development. Aqueous extracts of neem were also shown to enhance both humoral immunity and cell-mediated immunity (Ray et al. 1996). Neem leaf glycoproteins (NLGP) and extracts exhibit potential to enhance immunogenicity and block negative immunoregulatory host mechanisms in various tumours. Sarkar et al. (2008, 2010) reported that NLGP induces anti-tumour immunity by enhancing carcinoembryonic antigen (CEA) presentation of dendritic cells to T and B cells. In addition, NLGP inhibits T-regulatory cell (Tregs)-associated murine tumour growth by downregulating the expression of Foxp3, CTLA4, and GITR and facilitating reconditioning of the tumour microenvironment by increasing interferon-γ (IFN-γ) and IL-12 secretion (Chakraborty et al. 2011). Chakraborty et al. (2010) showed that NLGP exhibits anti-tumour activity in patients with head and neck squamous cell carcinoma by activating cytotoxic T lymphocytes and natural killer cells. Neem leaf preparations are also reported to enhance Th1-type immune responses and anti-tumour immunity against breast tumour associated antigen by inhibiting the release of IL-10 and promoting IFN-γ secretion (Mandal-Ghosh et al. 2007).

4.3.2.8 Epigenetic Alterations

Epigenetic modifications such as DNA methylation and histone acetylation play a major role in regulating the dynamics of gene expression. DNA methyltransferases (DNMTs), DNA demethylases, histone acetyltransferases (HATs) and histone deacetylases (HDACs) act as key mediators of the epigenetic processes. While CpG island methylation by DNMTs and histone deacetylation by HDACs result in repression of gene expression, histone hyperacetylation by HATs activates gene expression (Hassler and Egger 2012). The limonoids azadirachtin and nimbolide exhibit the potential to inhibit the expression of HDAC-1 and restrict tumour invasion and angiogenesis during DMBA-induced HBP carcinogenesis (Vidya Priyadarsini et al. 2009). HDAC inhibition by these limonoids is of particular significance in the perspective of the emerging interest in epigenetic reprogramming in cancer and the potential anticancer effects of HDAC inhibitors (Carew et al. 2008). Figure 4.1 summarizes the molecular targets of neem and its constituent phytochemicals.

4.4 Toxicity Studies

Several in vivo studies have demonstrated neem extracts and limonoids to be potentially safe. Aqueous extracts of neem leaf were non-toxic to mice up to oral doses of 1,000 mg/kg body weight (Biswas et al. 2002). Acute toxicity studies in mice have revealed that the LD_{50} value for methanolic neem leaf extract is 13 g/kg body weight (Okpanyi and Ezeukwu 1981). Azadirachtin at daily doses of 15, 0.26 and 0.3 mg/kg body weight was found to be potentially safe for human consumption (Boeke et al. 2004). Technical azadirachtin, a mixture of seven structurally related tetranorterpenoid isomers, is also reported to lack toxicity in rats (Srivastava and Raizada 2007). However, higher doses of azadirachtin ($LD_{50} > 5{,}000$ mg/kg bw) were found to exhibit acute toxicity (Raizada et al. 2001). Pillai and Santhakumari (1984) demonstrated that oral doses of nimbidin up to 100 mg/kg body weight are non-toxic. Intragastric administration of nimbolide was also found to be non-toxic to experimental animals. However, intravenous and intraperitoneal administrations were found to cause death in experimental animals by lowering the arterial blood pressure and inducing dysfunctions in the kidney, small intestine, pancreas, and liver (Glinsukon et al. 1986).

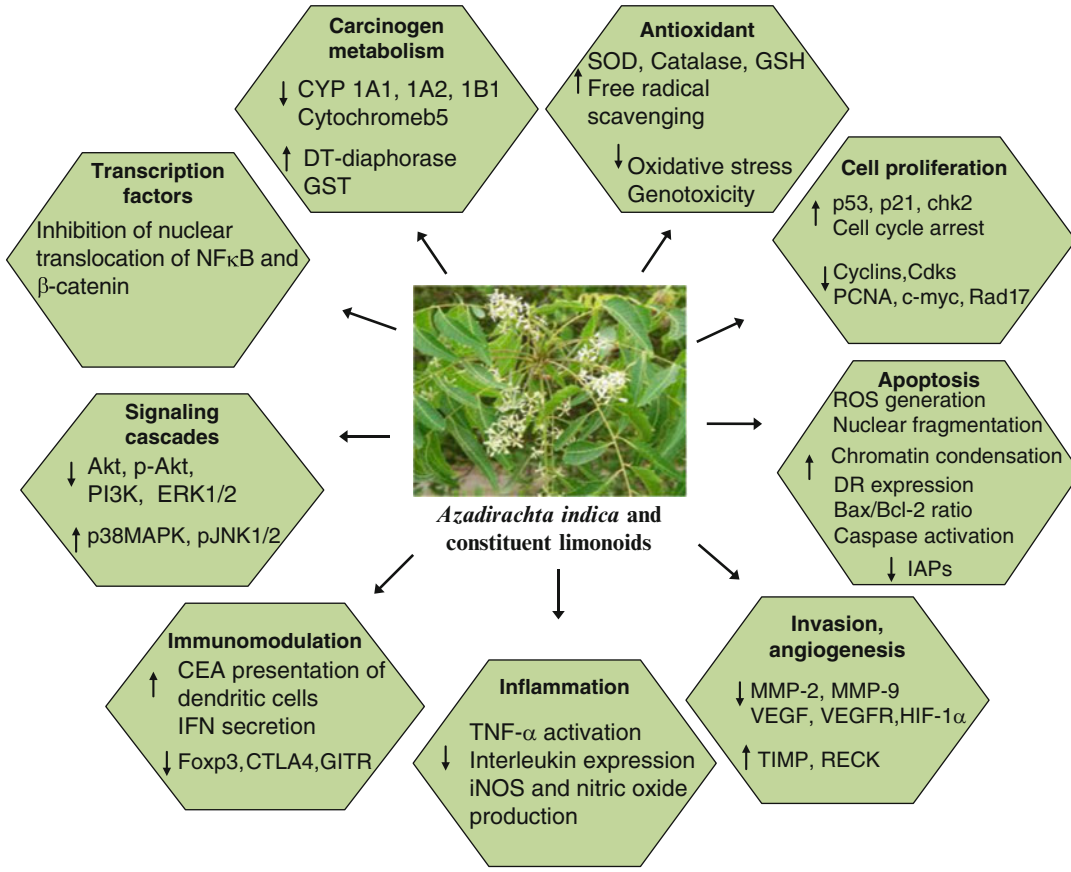


Fig. 4.1 Molecular targets of neem and its constituent phytochemicals

4.5 Conclusions and Future Perspectives

Globally, there is an increasing trend towards the use of natural products for medicinal purposes owing to their chemical diversity, intrinsic biologic activity, affordability, and lack of substantial toxic effects. Neem, a medicinal treasure of the Indian subcontinent with its wide array of phytochemicals is a promising candidate for anticancer drug development. Neem extracts and the constituent limonoids target multiple molecular and cellular pathways that are dysregulated in cancer including xenobiotic metabolism, cell cycle, DNA repair, apoptosis, matrix invasion, angiogenesis, immune surveillance, and intracellular signalling. However, the efficacy of neem extracts and limonoids has been tested only in preclinical models, and the cancer preventive and therapeutic potential in humans are largely unexplored. There is therefore a need for extensive investigations on the metabolism, pharmacokinetics, toxicity, precise molecular mechanism of action, expression profiling using high-throughput microarrays, and epigenetic remodelling in rigorous well-designed clinical trials for translating the beneficial effects of neem from the bench to the bedside.

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