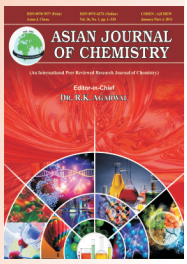
Asian Journal of Chemistry; Vol. 26, No. 16 (2014), 4940-4948

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16825



REVIEW

Terpenoids and Its Commercial Utility from Neem: The Nature's Own Pharmacy

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Received: 6 December 2013; *Accepted*: 4 February 2014; *Published online*: 28 July 2014; AJC-15606

Plant derived bio products have become integral part of world health care system which not only include botanical drugs but also agro chemicals, cosmetics, nutraceuticals, flavors and fragrances and so on. The World Bank estimates that global trade in plant derived products will grow at average 10-12 % per year and will reach whooping USD 123 billion by 2020. Inevitably this brought attention to our sthala vriksha or sacred tree neem (*Azadirachta indica*) considered as store house of high value products with vast array of biological activities like insecticidal, spermicidal, anticancer, hypoglycemic, antiulcer, antiinflammatory, and many more. However due to inherent complexities in scalability which includes consistent supply chain and difficulty in synthesizing bioactive metabolites using established chemical routes and seasonal variation in bio-efficacy with change in plant chemistry, place, age *etc.* makes large scale production of most of them is impossible. Hence there is need for a paradigm shift in combining traditional approaches with more sophisticated biotechnological methods to produce these products in heterologous system, thereby enabling a consistent scale-up at a commercial level. This article reviews the terpenoids reported in neem so far, their proposed biosynthetic pathways and potential avenues for expressing them in microbial cell factories.

Keywords: *Azadirachta indica*, Isoprenoids, Medicinal plants, Plant secondary metabolites.

INTRODUCTION

Azadirachta indica (neem), a versatile tree of immense value, is native to the Indian sub-continent belongs to the Meliaceae family has attained a pride of place in international scientific research and literature because of its diverse utility in pharmaceuticals, cosmetics, agriculture, veterinary, toiletries and as potential biomass[1] Therapeutic frequency index (TI) attributed to Neem is high and hence categorized under "elite species" due to its curative abilities in treating more than 25 critical human ailments and still currently being explored and widen the horizon to include FMCG, Agro and OTC products[2]. Neem tree, owing to its well-known therapeutic, pharmaceutical, agro, medicinal properties, is variously known as "Divine tree," "Heal all," "the wonder tree", "Nature's drug store," and "Village pharmacy"[3]. Diverse application of neem tree for mankind has been known since ages, as evident from its mention in various ancient Indian documents (Vedas, 2000 - 4000 BC) like '*Charak-Samhita'* and '*Susruta-Samhita*'. Indian system of natural treatment, Siddha and Ayurveda refers to neem tree as "*Kaya Kalpam*" (The name is from the Sanskrit language - kaya: body and kalpa: rejuvenation and "*Sarva Roga Nivarini*" (in Sanskrit, meaning "The curer of all ailments"), respectively[4].

Due to its plethora of biological activities it is the most researched tree in the world and is considered to be the most promising tree of 21st century[5]. In fact, scientific community believe that even half the qualities of this wonder tree hasn't discovered and every year there are additional reports for neem's new properties or chemistry. Almost every part of the tree such as fruits, seeds, leaves, bark and roots has long been used in traditional systems of medicines for the treatment of a variety of human diseases. *A. indica* is one of the most extensively studied plants globally and over 300 constituents have been isolated from its different parts and their structures were elucidated. Neem's extensive biological activities and FMCG applications (for example: cosmetics, toiletries *etc.*) are mainly due to its richness in secondary metabolites in which majority of them belongs to the class of isoprenoids[6].

Neem terpenoids current status: Since times immemorial, neem derived products have been used for food, medicine and agriculture leading to the common phrase "Nature's Pharmacy" for human welfare. Terpenoids are the majority among the secondary metabolites present in the neem (Fig. 1)

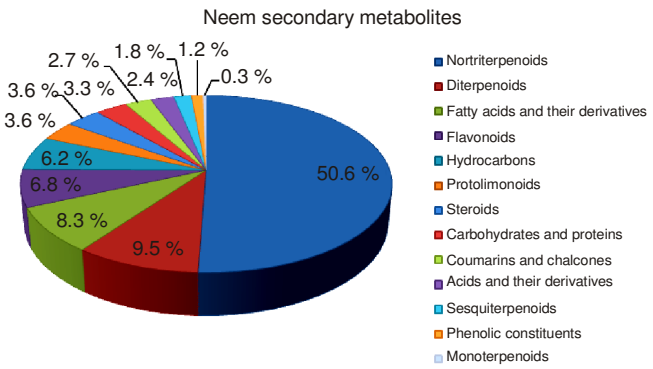


Fig. 1. Pie chart representing the major groups of neem secondary metabolites. Based on their numbers and diversity, terpenoids appeared to be capturing major portion and much potential in having assortment of industrial and medicinal applications

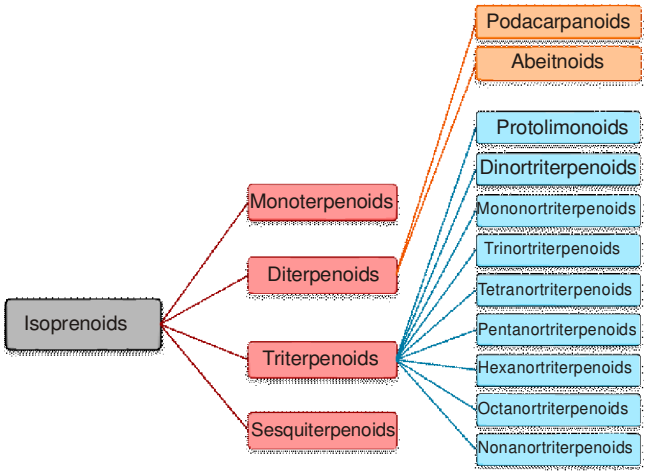


Fig. 2. Classification of Neem Terpenoids

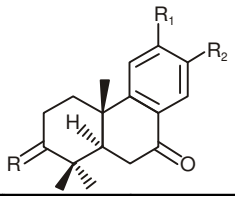
and they are categorized into various classes (Fig. 2) which are detailed in the following sections.

Elucidation of terpenoids from neem was dates back to 1942; Siddiqui was the first to report the isolation of nimbin, nimbidin and nimbinin. Since then more than 200 terpenoids have been characterized from different parts of neem and well-studied by various scientists in classifying these compounds.[7-8].

Monoterpenoids: Monoterpenoid, dihydromyrcenol is the first to be reported from the extract of neem fruit coats[9]. Other monoterpenes like α and β-Pinene, limonene, Myrcene, Camphene are also reported to be present in neem[10].

Diterpenoids: Podacarpanoids and abeitanoids are the two major groups of neem are reported so far. Twenty four compounds belonging to these groups (for example: sugiol, nimbiol, nimbosone, nimbilicin) are extracted from neem bark or root[11]. (Fig. 3).

Triterpenoids: Apart from β-amyrin which is a common triterpenoid present in all plants, many of neem plant specific triterpenoids are discovered and constitute the major group of isoprenoids. Based on current discovery status neem triterpenoids can be classified into nine groups[12]. *i.e.* Protomeliacians or Protolimonoids, Mononortriterpenoids, Dinortriterpenoids, Trinortriterpenoids, Tetranortriterpenoids, Pentanortriterpenoids, Hexanortriterpenoids, Octanortriterpenoids, Nonanortriterpenoids



Name	R	R_1	R_2
Azadirin A	H	Me	$(CH_2)_2OH$
Azadirin B	H	$(CH_2)_2OH$	Me
Margocilin	β-OH,H	OH	i-Pr
Margocin	O	H	i-Pr
Margocinin	O	OH	$MeCHCH_2OH$
Nimbidiol	H_2	OH	OH
Nimolinin	H_2	OH	Ac
Nimbisonol	β-OH,H	OH	Me
Demethylnimbionol	β-OH,H	OH	OH
Isomargolonone	O	COOH	Me
Margolone	H_2	Me	COOH
Margolonone	O	Me	COOH
Methylnimbiol	H_2	OMe	i-Pr
Nimosone	O	OH	i-Pr
Methylnimbionone	O	OMe	OMe
Nimbione	O	Me	OH
Nimbinone	O	OH	Me
Nimbionol	β-OH,H	OH	OMe
Nimbionone	O	OH	OMe
Nimbonone	H_2	Et	OMe
Nimbiol	H_2	OH	i-Pr
Sugiol	H_2	OH	Me

Fig. 3. Diterpenoids of neem (belong to two groups, podacarpanoids and abeitanoids)

Protomeliacians or protolimonoids: Protolimonoids (Euphol or Tirucallol derivatives) are thought to be biosynthetic precursor for meliacins or limonoids and so called protomeliacians[13].

Presence of twelve types of protolimonoids has so far demonstrated in neem plant (Fig. 4) in which meliantriol is one of the potent antifeedant molecule and is considered to be the first tetracyclic triterpenyl alcohol biogenetically related to tirucallol[14].

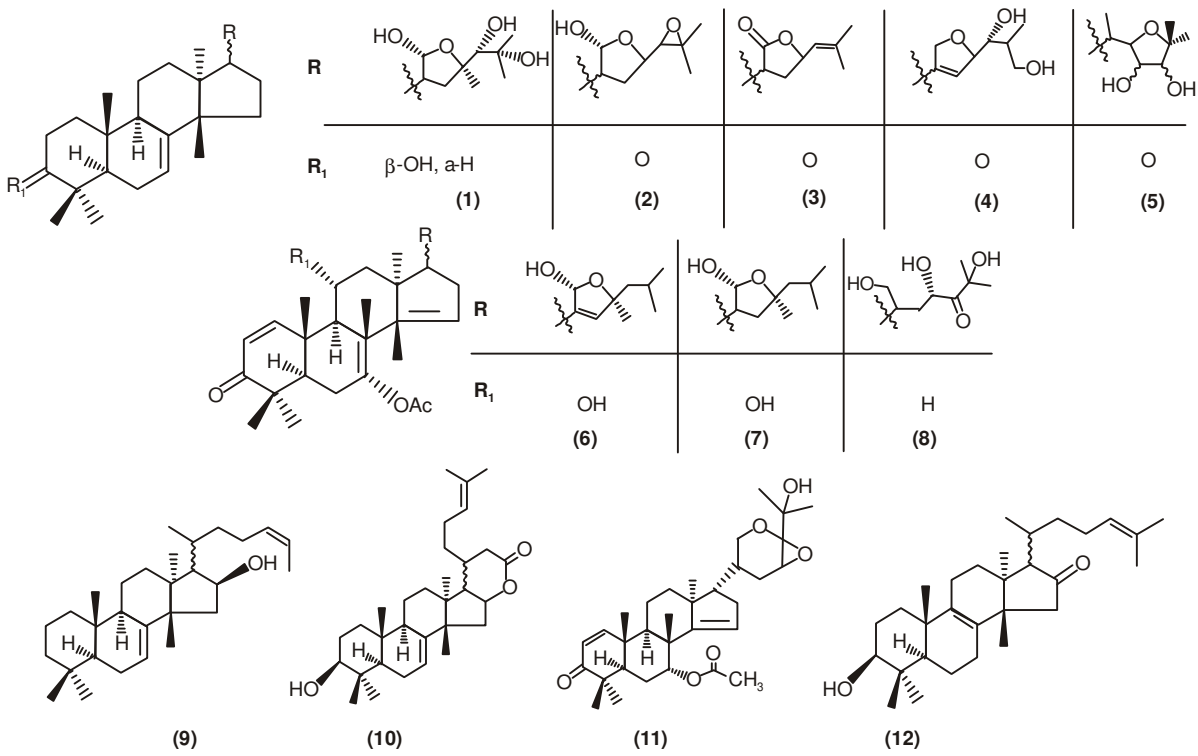
Mononortriterpenoids: Melianol (Fig. 5) is a ring C *seco*-mononortriterpenoid was isolated from neem leaves[15] and is the only one belong to this class was identified so far in neem plant.

Dinortriterpenoids: Three compounds azadirolic acid, azadiradionol and meliacinin (Fig. 5) belong to this class which is reported to be isolated from fruit coats[16].

Trinortrterpenoids: Limbonin, meliacinolactol and Zeeshanol are the ones considered to be part of trinortriterpenoids[17] (Fig. 5).

Tetranortriterpenoids: Tetranortriterpenoids are the major isoprenoids constitutes in large numbers with varied modified carbon chains in neem.

Azadirones: The unique feature of azadirone groups is the presence of oxygen function at C-3 and C-7 with no change in all the four rings of the basic skeleton. Meliacins of this family are further sub grouped into azadirone, azadiradione and epoxy-azadiradione in which oxidation reactions makes them related to each other[18] (Fig. 6).



1	Meliantrol	7	Azadirachnol
2	Melianone	8	Azadirol
3	Nimolinone	9	Limocinol
4	Nimbocinone	10	Kulactone
5	Odoratone	11	Diepoxyazadirol
6	Azadirachtol	12	Limocinone

Fig. 4. Examples for protolimonoids or protomeliacins

Two compounds 17-hydroxy azadiradione and 17-hydroxyl nimbocinol which are related to azadirones are reported to have toxicity to termites. Bactericidal activity was reported for epoxy azadiradiones like nimbin and epoxy nimbin[19].

Gedunins: Gedunin is one of the potent bioactive tetranorterpenoids of neem reported to a have anticancer, antifilarial, antifungal and antimalarial properties. The D-ring in parent structure of nimbinin, nimbicinol and 17 β and 17α hydroxyl azadiradione are considered to be oxidized probably by Baeyer Villiger type of reaction to give rise to gedunins[20] (Fig. 7).

Amoorstatin and vepinins: The biogenic precursor for these compounds could be nimocinol or other azadirones formed through addition of 17-OH to the C-14/15 double bond and formation of 7α and 17α-oxygen bridge. Amoorstatins possess a C-19/29 oxygen bridge and reported for its hypoglycemic activity[21] (Fig. 7).

Vilasinin: Vilasinin is considered to be a key intermediate in the biosynthesis of C-seco meliacins namely nimbin, salanin and azadirachtins. It is reported that vilasinins with C-12 substituent did not show considerable antifeedant activity as compared with vilasinin compounds with without C-12 substituent[22] (Fig. 7).

C-seco meliacins: Cleavage of carbon ring C of parent compound gives the name C-seco for these meliacins compounds. C-seco meliacins are the most important group of tetranortriterpenoids in neem having with structural complexity and numerous biological activities. One of the sub groups of C-seco meliacin family is potent insecticidal compound called azadirachtin which gives the uniqueness to entire neem chemical constituents[23,24]. Several analogs/derivatives of azadirachtin have been reported (Fig. 8) which includes isolation from neem and chemical modifications.

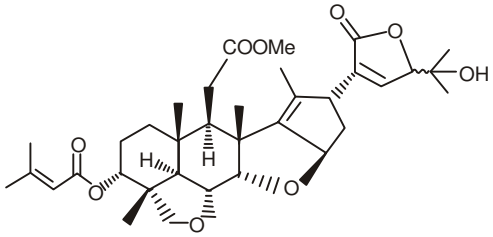
Pentanortriterpenoid: Nimbinene, 6-deacetylnimbinene, nimbandiol, 6-O-acetylnimbandiol and 4α-benzoylnimbandiol are the five compounds belong to this class were reported so far[25].

Hexanortriterpenoids: Nimolicinolic acid and nimbocin were isolated from fruits and root bark, respectively (Fig. 9). Nimolicinolic acid is considered to be the unique compound isolated from neem has apo-euphane or apo-tirucallane skeleton while other plants possess cucurbitacin, dammarane and lanostane skeletons[26].

Octanortriterpenoids: Four compounds desfuranoazadiradione, azadironol, desfurano-6a-hydroxyazadiradione and desfurano-desacetylnimbin were reported to belong to this class[27] (Fig. 9).

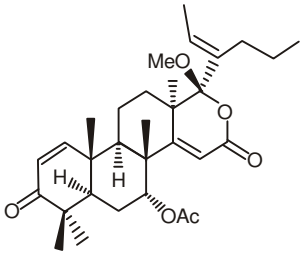
Nonanortriterpenoids: β-Nimalactone and α-nimalactone are the only two compounds reported to belong to this class[28] (Fig. 9).

Mononortriterpenoid

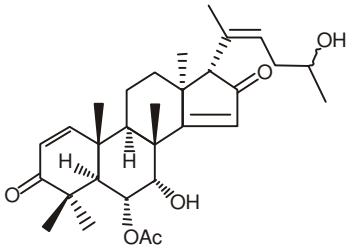


Melianol

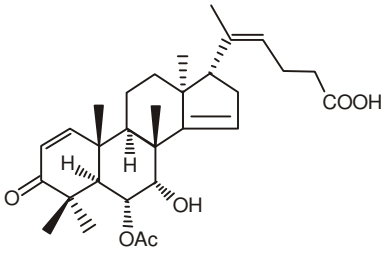
Dinortriterpenoid



Meliacin

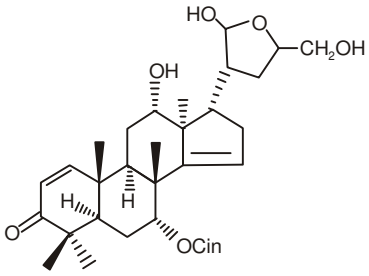


Azadiradionol

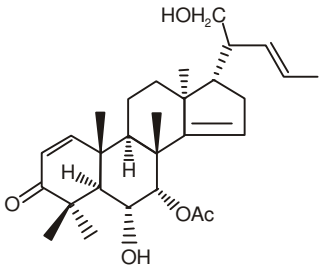


Azadirolic acid

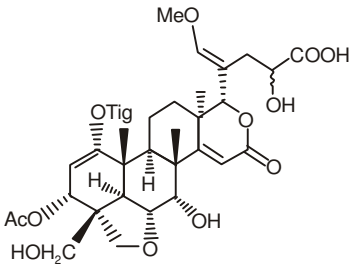
Trinortriterpenoid



Meliacinalactol

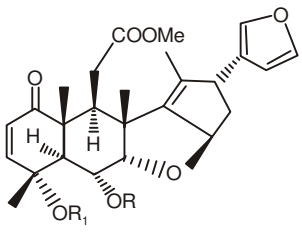


Zeeshanol

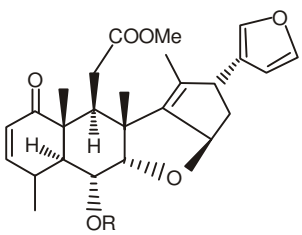


Limbonin

Pentanortriterpenoid

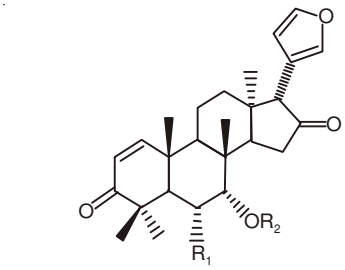


R	R_1	
H	H	(Nimandiol)
Ac	H	(6-O-acetylnimbandiol)
H	Bz	(4α-Benzoylnimbandiol)

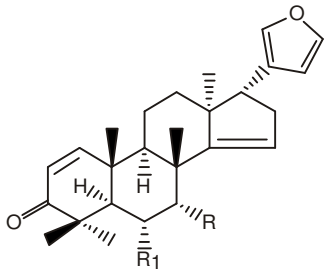


R	
H	(Nimbinene)
Ac	(6-Deacetylnimbinene)

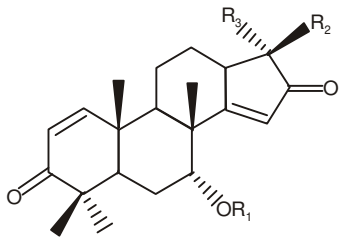
Fig. 5. Structures of mononortriterpenoids, dinortriterpenoids, trinortriterpenoids and pentanortriterpenoids



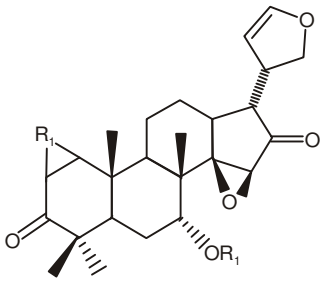
Name	R_1	R_2
Azadirone	H	Ac
7-Deacetyl azadirone	H	H
Nimocin	H	Bz
Nimocinol	OH	Bz
Meldenindiol	OH	H



Name	R	R_1
Meldenindiol	OH	OH
Meldenin	H	OAc
Isomeldenin	OAc	OH



Name	R_1	R_2	R_3
Azadiradione	Ac	H	β-Furan
17-Epi azadiradione	Ac	β-Furan	H
17-β-Hydroxy azadiradione	Ac	OH	β-Furan
17-α-Hydroxyl azadiradione	Ac	β-Furan	OH
Nimbocinol	H	H	β-Furan
17-β-Hydroxy nimbocinol	H	OH	β-Furan



Name	R_1	R_2
Nimbinin	Ac	Δ^1
Dihydro nimbinin	Ac	H,H
1b, 2β-epoxy nimbinin	Ac	1β, 2β-O

Fig. 6. Structures of azadirone family

Sesquiterpenoids: First sesquiterpenoid identified in neem was the methyl (2E, 6E)-farnesoate isolated from fruit coats. Various sesquiterpenoids identified from neem include β-farnesene, Caryophyllene, Germacrene B, Valencene and α-Himachalene[29].

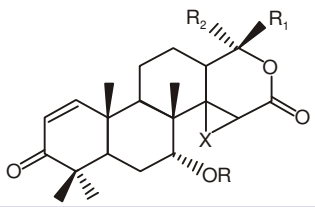
Full potential still largely untapped: Plant steroids and terpenoids based bio products account for USD 12.4 billion global market per annum[30]. According to leading market survey company BCC research, the global market for botanical and plant-derived drugs is expected to reach USD 26.6 billion in 2017 with a compound annual growth rate (CAGR) of 3.7 %.

All the parts of the neem tree find extensive application and usage in natural system of Indian medicines like Ayurvedha and Siddha. Modern day medicine has identified a wide range of activities from various parts of the neem tree. These actions include azadirachtolide: anti-hyperglycemic and anti-lipidemic, Nimbin and Nimbidin: anti-inflammatory, anti-pyretic, antihistamine, anti-arrhythmic, anti-fungal, anti-ulcer, Nimbidol: anti-tubercular, anti-malaria, Gedunin-vasodilator, sodium nimbinate: spermicidal, salanin: repellant *etc*[31].

Though neem has proved a promising tree, yet lot of research is required to prove its multiple uses to mankind. However the success rate for neem derived products into commercialization is low and promising products often languish through lack of volume generation because of numerous reasons. Large scale extraction of important secondary metabolites from neem suffers with lack in high purity coupled by lower yields and high cost, which in turn, leads to compromise in quality and bio efficacy.

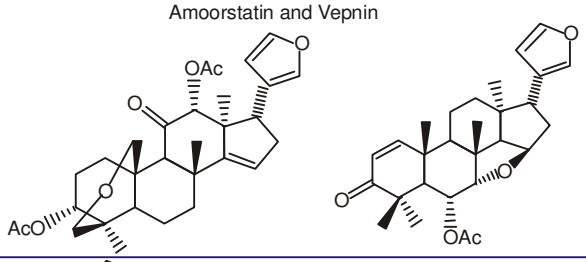
Another challenging arena is the huge batch to batch variability which in turn leads to lack of reproducible bio-activity. In addition, a scale up to address huge market demand requires a lot of biomass (plants) which in turn has a negative carbon footprint. Moreover, chemical synthesis of such molecules is often impeded by its long and protracted synthetic routes and also difficulty in separation due to several chiral centres. It took 22 years of hard work to synthesize azadirachtin[32]. Total number of steps to synthesize it chemically is 71 and the final yield is a meager 0.00015 %. This also has an impact on the affordability of such molecules for widespread

Gedunin

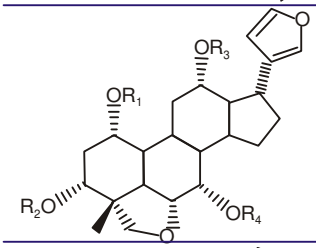


Name	X	R	R_1	R_2
Gedunin (Gn)	O	Ac	H	β-Furan
7-Deacetyl (Gn)	O	H	H	β-Furan
7-Deacetyl-7-benzoyl (Gn)	O	Bz	H	β-Furan
Nimolicinol	Δ^1	Ac	β-furan	OH

Amoorstatin and Vepnin

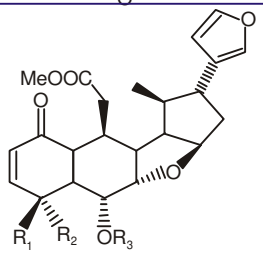


Vilasinin



Name	R_1	R_2	R_3	R_4
Vilasinin (vn)	H	H	H	H
1,3-Diacelyl vn	Ac	Ac	H	H
1-Tigloyl-3-acetyl vn	Tig	Ac	H	H
1-Tigloyl-3-acetyl vn 12-Acetoxy vn	Tig	Ac	H	OAc
1,3,7-Triacetyl vn	Ac	Ac	Ac	H

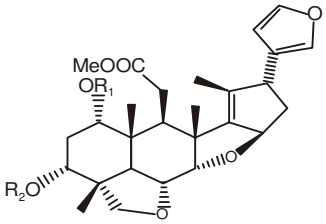
Nimbin



Name	R_1	R_2	R_3
Nimbin	CH_3	COOMe	Ac
6-Diacetyl nimbin	CH_3	COOMe	H
4-Epinimbin	COOMe	CH_3	Ac
6-Deaceiyl nimbinal	CH_3	CHO	H
Nimbinal	CH_3	CHO	Ac
Nimbinol	CH_3	CH_2OH	H
Nimbic acid	CH_3	COOH	H

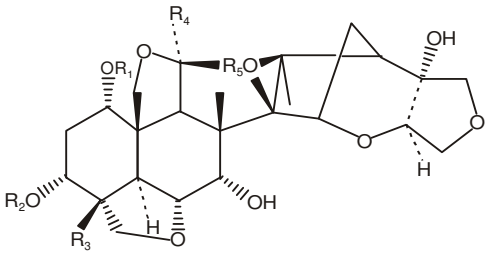
Fig. 7. Structures of some of tetranortriterpenoids class of compounds

Salannin



Name	R_1	R_2
Salannin	Tig	Ac
3-Deacetyl salannin	Tig	Ac
Salannol	ival	H
Salannol acetate	ival	Ac

Azadirachtin



Name	Aza symbol	R_1	R_2	R_3	R_4	R_5
Azadirachtin (aza)	A	Tig	Ac	COOMe	COOMe	OH
3-Deacetyl-3-cinnamoyl (aza)	–	Tig	Cin	COOMe	COOMe	OH
3-Tigtoyl azadirachtin	B	H	Tig	COOMe	COOMe	H
3-Tigtoyl azadirachtin	E	H	Ac	COOMe	COOMe	OH
Azadirachtol	–	H	H	COOMe	COOMe	H
Azadirachtin	D	D	Tig	Ac	Me	COOMeOH
Azadirachtin	I	I	Tig	Ac	Me	H OH
11-Demethoxycarbonyl (aza)	H	Tig	Ac	H	H	OH

Fig. 8. Salanin family is featured with two oxygen bridges, C-6/28 and C-7/14. Azadirachtin is the complex molecule with numerous oxygen atoms and it's a most potent insect anti-feedant

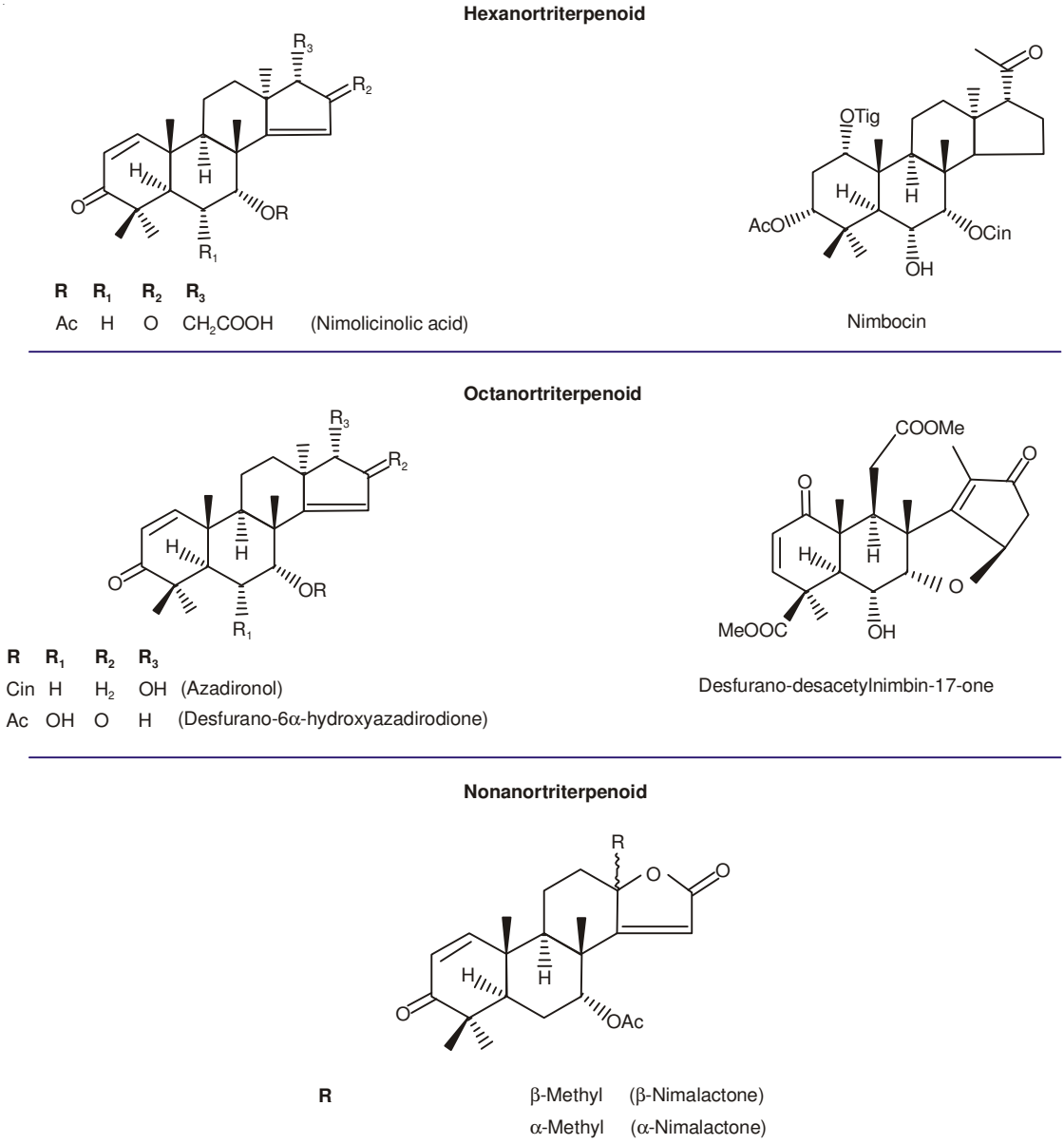


Fig. 9. Structures of hexanortriterpenoids, octanortriterpenoids and nonanortriterpenoids

therapeutic application. Formulation based large scale commercialization also not able to deliver at affordable prices because of fragile natural source of supplying seeds with reliable terpenoid content and extraction[33].

In order to effectively address these challenges, production of neem terpenoids in heterologous systems will ensure a stable supply chain at affordable prices. Despite decades of research, genes involved in neem secondary metabolism remains poorly characterized. Development of neem plant genome and trancriptomics is still in budding stage. Recently genome and transcriptome sequencing of neem have been reported indicating the discovery of potential genes involved in terpenoid biosynthesis[34]. However genetic maps of biosynthetic pathways are still far from complete, whereas knowledge on the regulation of these pathways is practically nonexistent. As a result, the diversity of genes encoding enzymes involved in neem's secondary metabolism is not well represented, or at least not well annotated in public DNA sequence databases.

Neem is known to produce a bewildering array of specialized terpenoid metabolites based on a myriad of skeletal isoprenoid structures and functional group combinations. Given their enormous structural diversity and the equally staggering numbers of terpenoid molecules produced, the impressive biosynthetic pathways for these molecules are yet to be tapped. Functional genomics based deciphering of the biosynthetic pathways/genes for neem terpenoids could be the key issue for its industrial production.

Harnessing enzymes from neem to engineer terpenoids with commercial potential: Elucidation of entire pathway for neem terpenoid pathway genes can be used for metabolic engineering. Also the entire biosynthetic pathways could be transferred to other hosts such as *E. coli* or yeast to produce desired molecule in industrial scale. Gene or pathway information for neem terpenoid biosynthesis is sparse however based on radiolabel based studies, it was postulated that azadirachtin biosynthesis precursors are derived from mevalonate pathway in which cyclization of squalene results into

tetracyclic ions euphane/euphol and tirucallane/tirucallol two chemically similar compounds[35].

Euphane/tirucallane is the first biogenic precursor formed from cyclization of squalene which is then further cyclized and addition of furan ring is catalyzed by the unknown enzymes to give rise to limonoids such as azadirone and azadiradione. Allylic isomerization of euphane/tirucallane give rise to butyrospermol, which is then oxidized and rearranges through a Wagner-Meerwein (W-M) reaction by 1,2 methyl shift to form apo euphane/tirucallane. Cleavage reactions of four terminal carbons on the side chain leads to formation of a tetranor-triterpenoid intermediate (Fig. 10) and upon cyclization of remaining carbons on the side chain give rise to furan ring which is finally oxidized to form azadirone and azadiradione. C*seco*-limonoids such as nimbin, nimbidinin and salannin are formed from either of azadirone or azadiradione molecules through oxidation and esterification with a molecule of tiglic acid, a derivative of L-isoleucine. It is postulated that azadirachtin and related analogues are biosynthesized upon further oxidation and cyclization of azadirone and salanin[36].

Terpenoid cyclases or synthases catalyzes the formation of parent scaffolds of the various monoterpene, sesquiterpene and diterpene types from these key precursors. Terpenoid synthases catalyzes the intermolecular electrophilic coupling reaction with various cyclizations and rearrangements, including hydride shifts, methyl migrations and Wagner-Meerwein rearrangements, which largely contributes to the great structural diversity and vast array of terpenoids. Subsequent biosynthetic steps involving terpenoid skeleton modifications like oxygenation or hydroxylation are often catalyzed by cytochrome P450s (CYPs)[37]. Thus the biosynthesis of neem terpenoids involves very complex oxidation and cyclization reactions

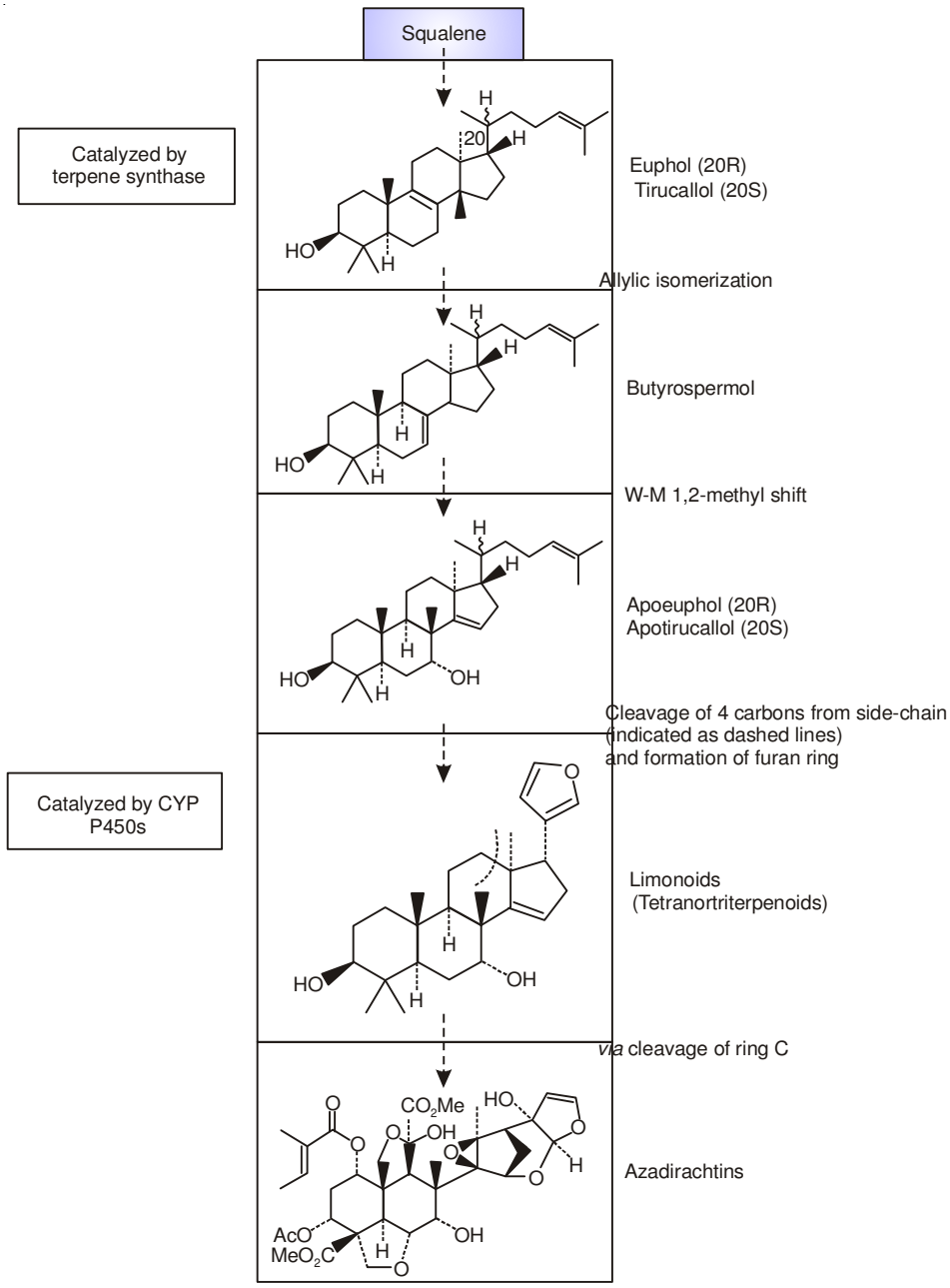


Fig. 10. Proposed biosynthesis pathway of azadirachtin and other related limonoids

presumed to be catalyzed by series of enzymes namely terpenoid synthases or cyclases and CYPs. In neem, biosynthesis of euphane/ tirucallane which is the primary precursor for limonoids could be possibly catalyzed by terpene synthases. Multifunctional CYPs then modifies euphane/ tirucallane to form several terpenoids.

Terpenoid biosynthesis has been well established in various host systems like *Escherichia coli*, or *Saccharomyces cerevisiae* or insect cells. For example, successful demonstration of terpenoid pathways like taxol and artemisininin in hosts other than plants has led to large-scale microbial production[38]. Production of amorphadiene up to 24 mg/L was achieved in *E. coli*[39]. Taxadiene was successfully biosynthesized in *Saccharomyces cerevisiae* by combining eight genes out of ten of the taxol biosynthetic pathway[40].

With advent of bioinformatics tools combines with enzymology and protein chemistry, production of potential neem metabolites in heterologous system is now feasible.

Conclusion

Considering the rapid advances in neem terpenoid knowledge and potential commercial uses, it is conceivable that genes involved in secondary metabolite biosynthesis of this plant to be explored. With advent of next generation sequencing facilities in its fold, it is now possible to integrate modern genomic tools with existing data to dissect the metabolic pathways and the role of key genes associated with their regulation. Neem pathways/genes can then be subject to new metabolic engineering efforts and applications.

High value neem compounds produced in heterologous hosts like *E. coli* or *Saccharomyces cerevisiae* which is considered as generally recognized as safe (GRAS) offers the potential for increased functionality and quality at lower production costs, including metabolites of great significance to human welfare [as drugs, fast-moving consumer goods (FMCG), nutarceuticals, cosmetics, over-the-counter (OTC)] such as anti-spermicidal, antiviral, anticancer, antibacterial, anti-inflammatory, anti-ulcer antimalarial, bio pesticides *etc*. which are of utmost relevance to Indian sub-continent. Also the production of high vale neem products in a non-plant host has huge impacts on sustainability and the protection of the environment, human health, industrial competitiveness and capable of generating growth.

ACKNOWLEDGEMENTS

The authors thank the Management of Dalmia Centre for Research & Development Coimbatore and Evolva Biotech Pvt. Ltd, India for providing necessary research facilities.

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