Chemical and biological investigations on *Azadirachta indica* (the neem tree)

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The isolation of azadirachtin, the potent insect anti-feedant and ecdysis inhibitor from the fruit kernels of neem (Azadirachta indica) has aroused world-wide interest in this tree, due to the promise of providing a non-toxic insect control agent for use in agriculture. Important aspects of the chemical, entomological and pharmacological work carried out on A. indica are presented in this article.

The neem tree (*Azadirachta indica* A. Juss, Family Meliaceae) has attained world-wide prominence in recent years[1]. Three international conferences[2] have been held—two in Germany and one in Kenya—for discussing the chemical and biological work carried out on the constituents of this tree. A recent monograph[3] *Focus on Phytochemical Pesticides* is devoted entirely to the neem tree. Another monograph[4] *Insect Growth Regulators* deals extensively with the growth-regulating activity of some neem-derived products. Over 2400 plants are known to elaborate insecticidal and insect-repellent constituents[5], but only neem holds out the promise of providing a highly effective, non-toxic and environmentally harmless means of controlling or eliminating insect pests which inflict losses in agricultural production.

The neem tree is indigenous to South Asia. It is found in most parts of the Indian subcontinent in the tropical and subtropical, semi-arid to wet tropical regions. Indian settlers have been responsible for its introduction to the African countries, where it is abundant in the tropical belt from Somalia in the East to Nigeria, Mauritania, Togo, etc. in the West. Introduced first into Fiji, it has spread to many islands in the South Pacific. From Trinidad, it has spread to other islands in the West Indies and many countries in Central and South Americas. It is being raised in Puerto Rico, Florida and Southern California. Large-scale plantations have come up in Malaysia and the Philippines as a source of timber and fuel wood. A grove of 50,000 trees has been planted outside Mecca for affording shade to pilgrims[6]. Thus the neem tree has spread to many parts of the world, in the tropical and subtropical regions, where there is no frost.

Various parts of the tree have been in use in India for several millennia for medicinal purposes and Ayurveda regards the tree as a 'sarva rogha nivarini'. The decoction of the bark is prescribed for fever, rheumatism, lumbago, etc. The oil is used in treatment of tetanus, urticaria, eczema, scrofula, erysipelas and in the early stages of leprosy. Neem leaf juice is used for expelling worms and curing jaundice and skin diseases. Neem twigs are employed in many village communities as a disposable tooth brush and help preserve healthy teeth. A branch of neem leaves is employed by the village poojari (priest) even today for driving off evil spirits! The breeze blowing on the neem tree is supposed to be beneficial to persons living in the vicinity and the tree is greatly cherished by Indians.

Neem leaves kept between folds of woollen or silk clothing is claimed to preserve them from insects. Before the era of synthetic insecticides, aqueous neem kernel extracts were used for warding off insect attack on crops. During the past four decades, the Khadi and Village Industries Commission has been responsible for promoting the use of the non-edible neem oil in soap-making. The oil instead being of limited use in medicine has become an important industrial product and neem seeds are being processed in a large number of oil-extraction units, especially in the Southern states. Neem cake obtained after removing the oil is in great demand as a fertilizer, since it kills root nematodes and helps lessen insect attack.

According to a survey[7] conducted in 1959, there were about 14 million neem trees in India, more than half of this in UP and the rest in Tamil Nadu, Karnataka, Andhra Pradesh, Maharashtra and Gujarat. A fully grown tree (about 10 years old) can yield about 50 kg of seeds and half a million tons of seeds should be available annually. Fully mature fruits drop from the trees. Birds relish the sweet mucilaginous portion of the fruits and reject the bitter skin and the seed. Most of the neem trees in India have come up by seed dispersed by birds. The collection of seeds extends from July to September and affords some income to the rural poor, who collect the seeds and deliver to the collection

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centres in their vicinity. It is unfortunate that no attempt has been made in India to raise large plantations, even though the tree can flourish in arid and semi-arid regions, in poor soils, is quick growing and can withstand drought and high temperatures. However the neem tree is being used as an avenue tree in many housing colonies of industrial units.

Chemistry of neem constituents

Siddiqui[8] was the first to isolate a crystalline compound, nimbin, from neem oil, blossoms, root and trunk bark. The structure of nimbin was solved many years later by workers in India[9-12] and by Overton *et al*[13]. After the work on nimbin, interest in neem constituents became dormant, but for the report by Narayanan's group on the isolation of vilasinin[14] and vepenin[15].

In the early sixties some papers were published[16] in India, reporting that neem seed kernel extract showed a strong anti-feedant effect on the desert locust (*Schistocerca gregaria*) and on the migratory locust (*Locusta migratoria*). Many years earlier, a similar report had been made[17] on the anti-feedant activity of leaf extract of *Melia azedarach* L., a tree closely resembling neem. The reports from India came when world-wide concern had developed on the harmful effects of most synthetic insecticides on mammals and the environment. Plants and insects have co-existed for hundreds of millions of years and plants have evolved strategies of keeping insects at bay, by producing secondary metabolites, which are insect-repellent or insecticidal. It was hoped that non-toxic compounds could be isolated from plants suited for insect-control.

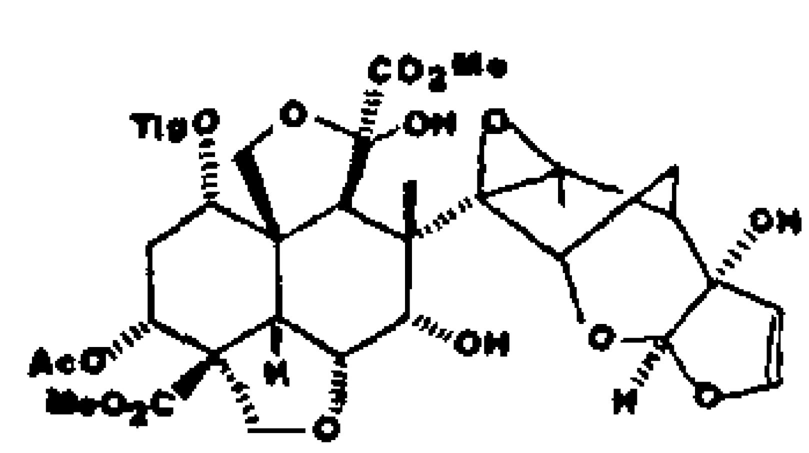
Isolation of azadirachtin

It is in this context that the isolation of azadirachtin from neem kernel extract by Butterworth and Morgan[18] was a giant leap forward in this quest. Careful chromatographic fractionation of neem kernel extract, monitored by anti-feedant assays with *S. gregaria*, led to the isolation of azadirachtin as a microcrystalline compound with astonishing activity against insects. Extensive work on the compound has established that it inhibits feeding in a variety of insects (over 200 species) at a concentration of 10–100 ppm). Even more interesting, is that it exhibits ecdysis inhibition activity at much lower concentrations, 1–10 ppm. This prevents the insect larvae from developing into mature insects, which could further multiply and produce new generations. Diminishing the fecundity of harmful insects is an excellent way of improving crop yields.

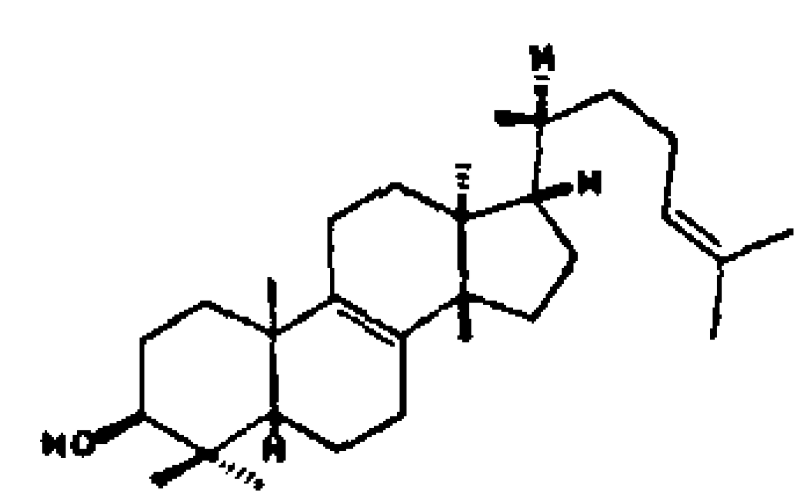
Several other procedures of isolating azadirachtin have been subsequently reported, which are essentially the same, but for variations in the adsorbents and solvent systems in the chromatographic operations[19-23]. In a more recent method the application of direct preparative HPLC[24] without prior column chromatography eliminates tedious procedures for obtaining azadirachtin. An improved method reported[25] subsequently affords pure azadirachtin, m.p. 160° $[\alpha]_D^{25} = -66°$ ($CHCl_3$, $C = 0.5$) cf. (ref. 29b).

Elucidation of structure of azadirachtin

Morgan[27] established the correct molecular formula of azadirachtin as $C_{35}H_{44}O_{16}$ and delineated important structural features in the molecule. In 1975 Nakanishi *et al.*[27] made a bold proposal for its structure, by application of newer NMR methods, particularly ^{13}C studies. However there were many features in this structure not consistent with the NMR data. Renewed efforts were made by the groups of Ley[28], Kraus[29a,b] and Nakanishi[30] which were considerably facilitated by the availability by this time of high-field NMR spectrometers and a range of new techniques possible with these instruments. Kraus's group succeeded in proposing the correct structure (**1**) which was confirmed by X-ray crystallographic analysis[31] of detigloyldihydroazadirachtin which happened to be crystalline.



(**1**) Azadirachtin



(**2**) Tirucallol

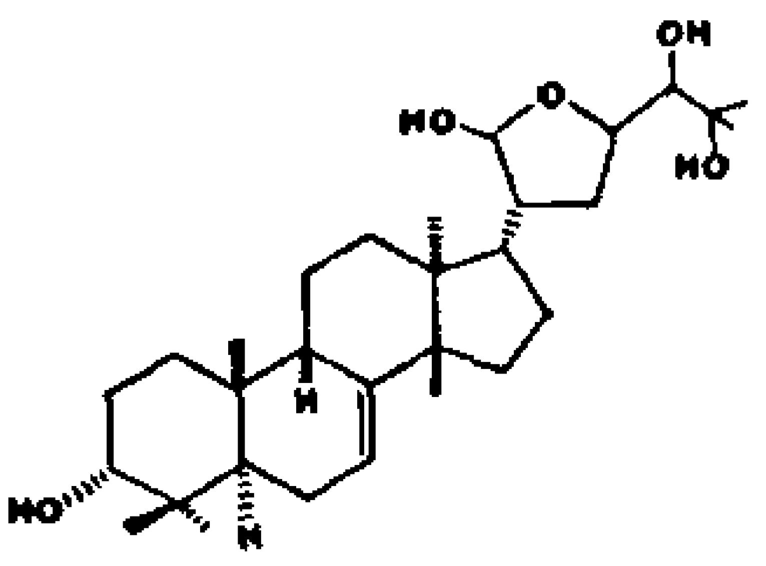
Other triterpenoids of *A. indica*

The discovery of azadirachtin with its remarkable biological activity gave a fresh impetus to the chemical investigation of neem and every part of the neem tree has been examined and over a hundred new compounds have been isolated by the groups of Kraus (Germany), Lavie, Ley, Connolly, Overton (England) and Siddiqui (Pakistan) among others. The structures have been established by employing high-field NMR and mass spectrometry and in some cases by X-ray crystallography. There are several excellent reviews[32,33] on compounds isolated from neem.

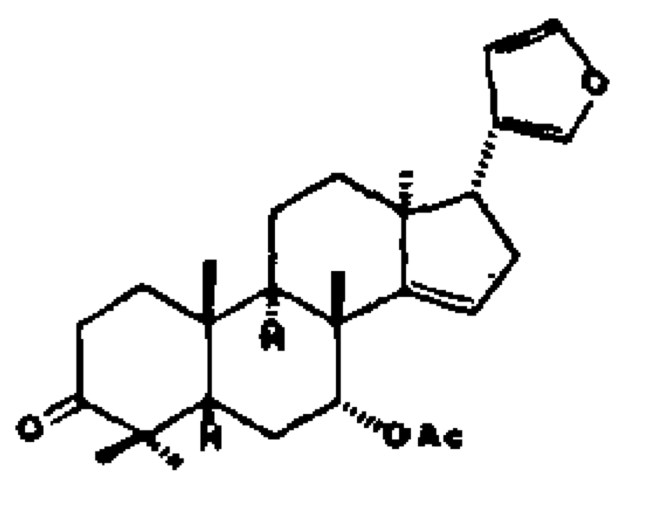
The most interesting compounds from neem are from the kernels and the well-characterized compounds are triterpenoids, which all seem to be derived from the tetracyclic triterpene tirucallol (**2**), as can be inferred from the stereochemical arrangement of the methyl groups at C-10, C-13, C-14 and the side chain at C-17.

The compounds occurring in neem kernels arise presumably by successive rearrangement and oxidation of the parent triterpene. There seems to be a progression of compounds to higher oxidation states from nimbin to salannin to azadirachtin as the seeds mature. The compounds occurring in mature neem kernels fall into several distinct groups.

There are a few triterpenoids, in which the side chain is intact as in meliantriol (3).



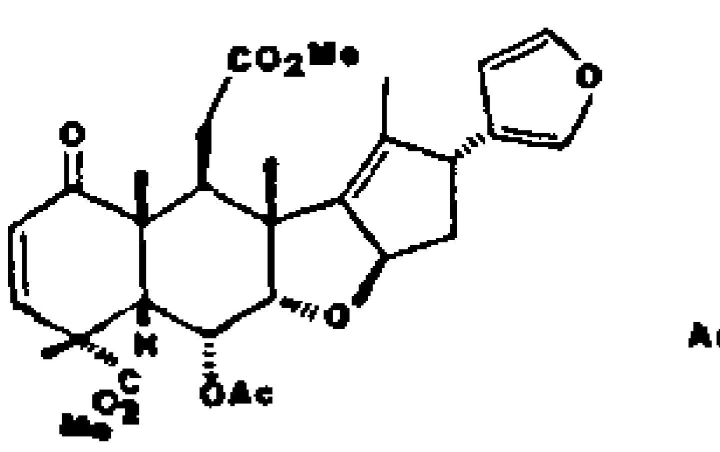
(3) Meliantriol



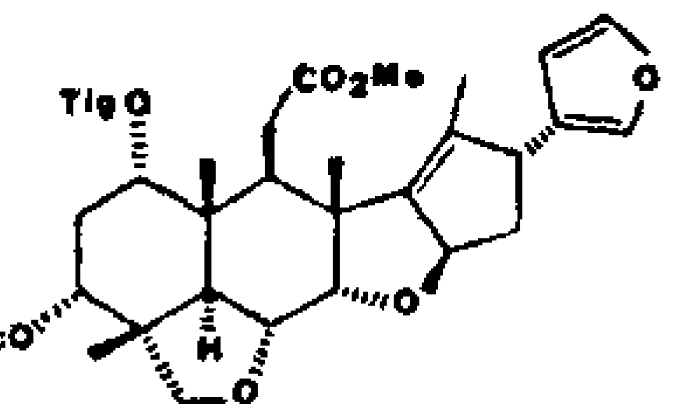
(4) Azadirone

There are a large number in which four carbon atoms C–24 to C–27 have been cleaved from the side chain leading to the tetranortriterpenoids. Of these, those with the remaining side chain carbon atoms cyclised to a furan ring are termed limonoids (meliacins), of which there are about thirty compounds, an example being azadirone (4).

There are several limonoids in which the A, C or D rings have been cleaved. The best example is nimbin (5), the first crystalline compound obtained from neem, in which the C-ring has been cleaved. There are many variations of nimbin, produced by further oxidation such as salannin (6), nimbolide, nimbandiol, etc.



(5) Nimbin

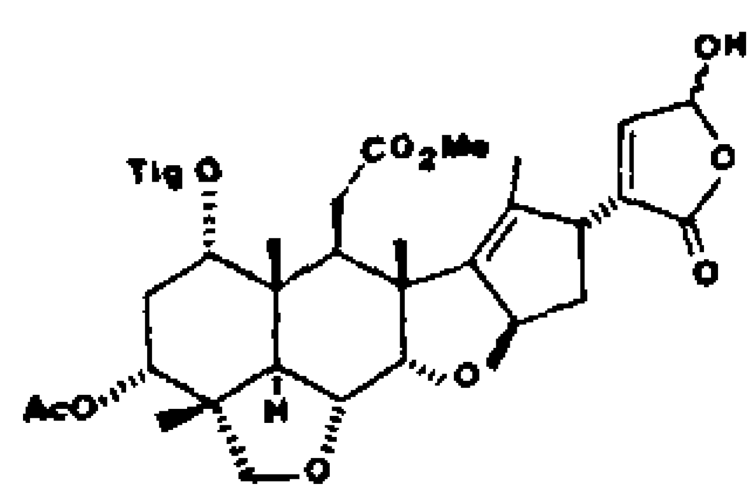


(6) Salannin

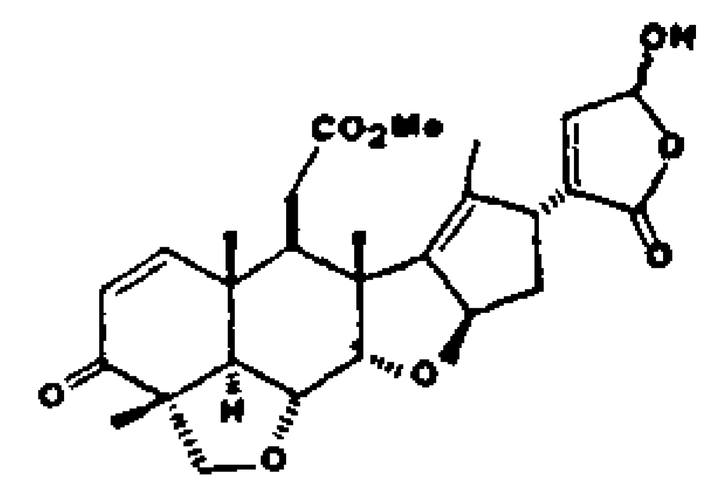
Azadirachtin (1) and its congeners represent yet another type of tetranortriterpenoid, distinct from the limonoids. These are characterized by the presence of a 1,3-dioxygenated A-ring, and at the other end of the molecule, a dihydrofuran (or modified) system. Several close congeners of azadirachtin are also present[29b] in neem kernels, such as 3-deacetyl-3-cinnamoylazadirachtin, 1-tigloyl-3-acetyl-11-methoxyazadirachtin, 22,23-dihydro-23-β-methoxyazadirachtin, 3-tigloylazadirachtol as well as the corresponding 1-tigloyl isomer[34].

Rembold[35] subjected neem kernel extract enriched in azadirachtin to preparative HPLC and obtained, besides Morgan's azadirachtin (termed A) and Kraus's 3-tigloylazadirachtol (B), five other compounds which were named azadirachtins C–G, for which structures (with the exception of C) were assigned on the basis of NMR spectra. The chromatography was monitored by the *Epilachna* bioassay, which has been developed as a fine tool in measuring growth-inhibition activity of several of the compounds isolated from neem kernels. Three other compounds, azadirachtins H, I[36] and K[37] have also been obtained by preparative HPLC and structures assigned on the basis of mass, ^1H and ^{13}C NMR spectrometry. All the azadirachtins A–K display high degree of activity in the *Epilachna* bioassay[38]. Neem kernels appear to be a veritable cornucopia of triterpenoid compounds and more than fifty compounds have been isolated from this source alone.

Other parts of the neem tree such as tender and mature leaves, flowers, fruits, fresh twigs, stem bark and root barks have also been investigated. Salannolide[39] (7) from the oil and margosinolide[40] (8) from the fresh twigs[40] represent ring-C-seco-tetranortriterpenoids, with τ-hydroxybutenolide side chain. Penta[41]- and hexanortriterpenoids[42] have also been isolated. Non-terpenoid compounds isolated from different parts of neem include hydrocarbons, fatty acids, steroids, phenols, flavanoids, glycosides, sugars, amino acids and sulphur compounds.



(7) Salannolide



(8) Margosinolide

Neem kernels contain 30–40% oil and 2.5–3% triterpenoids. Azadirachtin A is by no means a minor component of neem kernels. Material procured from several centres in S. India contains 0.2–0.3% azadirachtin on the average, whereas material from W Africa (Togo) is reported to contain 0.6%. The yield of azadirachtin varies greatly with the soil, the climate, maturity of the seeds and length of storage. Lack of uniformity of azadirachtin content is the most striking feature of neem kernels procured from various centres and even from the same centre at different periods, which, of course, is of no consequence for the production of the oil and the cake, which are in great demand.

Besides azadirachtin and its congeners, there are other compounds isolated from neem such as meliantriol, salannin, 7-desacetyl-7-benzoylazadirone, 7-desacetyl-7-benzoylgedunin, cis-(β-epoxy) azadiradione and 17-β-hydroxyazadiradione which have insect growth-disrupting properties, although not to the extent of the azadirachtins.

Biological activity of neem constituents on insects

An excellent summary of the biological activity of neem constituents on insects has been presented by Schmutterer[43]. Most of these studies have been carried out with neem seed kernel extract (NSKE) and very few with pure compounds like azadirachtin. There are obvious limitations in studies using NSKE, in which there are bound to be variations in the active principles and their relative proportions. However, some important conclusions could be drawn. The studies have covered the major orders of insects, *Orthoptera*, *Heteroptera*, *Homoptera*, *Hymenoptera*, *Coleoptera*, *Lepidoptera* and *Diptera*. Certain highlights from Schmutterer's conclusions are:

1. The development of immature stages is delayed after treatment at various stages of larval growth
2. Reduced uptake of food, which may delay post-embryonic development
3. High mortality between molts
4. Post-embryonic development disturbed, leading to various degrees of incomplete ecdysis
5. Treated instars may become permanent larvae which are unable to molt
6. Fecundity is decreased
7. Egg sterility.

The overlapping of anti-feedant effects with growth-regulatory activities account for the disturbance of metamorphosis and reduction of fecundity. Extensive studies have been carried out on the mode of action on insects employing pure azadirachtin. One recent hypothesis is that the release of neuropeptides disrupts the control of insect metamorphosis and behaviour on the level of molting, leading to inhibition of ecdysis. Another view is that azadirachtin blocks receptors for ecdysteriods which are needed for larval development. For further discussion on the mechanism of action of azadirachtin reference should be made to Schmutterer's review.

In a practical sense we are mainly concerned about agricultural pests which reduce crop yields. Table 1 gives a list of insect pests which are controlled by neem extracts.

Table 1. Agricultural pests controlled by neem

Pest	Common name
Callosobruchus maculatus (F.)	Cowpea weevil
Diabrotica undecimpunctata Mann.	Spotted cucumber beetle
Dicladispa armiger (ol)	Rice hispa
Epilachna varivestis Muls.	Mexican bean beetle
Leptinotarsa decemlineata (Say)	Colorado potato beetle
Rhizopertha dominica (F.)	Lesser grain borer
Sitophilus oryzae (L.)	Rice weevil
Tribolium castaneum (Herbst)	Red flour beetle
Trogoderma granarium Everts.	Khapra beetle
Atherigona soccata Rond.	Sorghum shootfly
Liriomyza sativae Blanchard	Vegetable leafminer
Orseolia oryzae (W. -M.)	Rice gall midge
Dysdercus koenigii (F.)	Red cotton bug
Bemisia tabaci (Genn.)	Whitefly
Nephotettix virescens (Dist.)	Green rice leafhopper
Nilaparvata lugens (Stal)	Brown planthopper
Cnaphalocrocis medinalis (Guen.)	Rice leaf folder
Heliothis armigera (Hb.)	Cotton bollworm
Earias insulana (Boisd)	Spiny bollworm
Lymantria dispar (L.)	Gypsy moth
Manduca sexta (L.)	Tobacco hornworm
Pectinophora gossypiella (Saunders)	Pink bollworm
Phthorimaea operculella (Zeller)	Potato tuber moth
Plutella xylostella (L.)	Diamond-back moth
Spodoptera litura (F.)	Tobacco caterpillar
Locusta migratoria migratorioides (R. &F.)	Migratory locust
Schistocerca gregaria (Forsk.)	Desert locust

Pharmacology of neem constituents

In traditional Indian medicine, neem products have been used for treatment of a whole gamut of diseases ranging from skin infections, cardiovascular disorders, diabetes and cancer. It is not surprising that extensive pharmacological and chemical work has been conducted to verify these claims. An exhaustive summary is provided by Jacobsen[44].

An extract made from dried neem leaves was found to cure eczema, ring worm and scabies in clinical evaluation tests. Neem leaf extracts had a marked anti-pyretic effect[45]. Nimbidinic acid[46], a component of neem seeds, was shown to be a potent diuretic[47]. Sodium nimbidinate at 10 mg/kg produced a perceptible fall in blood pressure in anaesthetized rats[48]. Neem seed oil was useful in relieving tissue oedema in congestive heart failure[49]. Neem oil and nimbidin, a bitter principle from neem, fed to fasting rabbits produced significant reduction of blood glucose level[50], half as active as tolbutamide. An aqueous extract of neem leaves was found to produce hypotensive activity with a minimal negative chronotropic effect as well as weak anti-arrhythmic activity[51]. Nimbidin gave significant protection from gastric and duodenal lesions in animals and enhanced the healing process in acetic acid-induced chronic gastric lesions in rats and dogs[52]. A patient with parotid tumours and another with epidermal carcinoma were successfully treated with injections of neem oil[53]. Extensive work has been done on the anti-fertility effects of neem oil, sodium nimbidinate and neem-leaf extracts. The contraceptive effects of neem oil administered subcutaneously have been well established[54]. The results of the various pharmacological tests carried out on neem constituents show that there is considerable merit in the use of these products in traditional Indian medicine.

Whether these pharmacological results can be the basis for developing modern drugs is a moot point. Most of the experiments have been carried out using nimbidin and nimbidinic acid which are amorphous materials, or on the leaf extract or neem oil containing many components. It is not known which component or components are responsible for the various biological activities. Much more work will have to be done tracking down the active principles, whether for use in circulatory disorders, diabetes, inflammation, cure of ulcer, etc., before any of them can be developed for use in modern medicine. Some progress has been achieved in the use of neem oil as a spermicidal or anti-implantation agent and this will probably be developed for use in population control programmes soon.

Use of neem products in agriculture

The use of neem kernel extracts for controlling insect infestation has been known in rural communities in India for a long time. With the advent of synthetic pesticides, the halogen compounds, organic phosphates, synthetic pyrethroids, carbamates, etc. with their instant and spectacular 'kills' old usages were given up. However, in the West, ever since the publication of Rachel Carson's *Silent Spring* thirty years ago, there has been continuing and ever-increasing concern over the use of synthetic pesticides and the harmful consequences, such as the simultaneous destruction of beneficial insects and the insect predators of pests and the development of resistance to pesticides, demanding higher doses and more frequent applications, increasing the costs and harming the environment. The method of application such as spraying of large farms by aeroplane or helicopter puts entire neighbourhood communities in jeopardy[55]. The general population is also subjected to the risk of toxic residues in the agricultural produce. A sense of urgency has imbued many scientists in the West, with a keen desire to develop less harmful methods of insect control.

In India, on the other hand, pesticide use is relatively small compared to the West or Japan, more probably due to costs involved, rather than a desire to save the environment. Although Indian agricultural scientists had indicated the powerful anti-feedant activity of neem kernel extract, chemists in India failed to comprehend the importance of these findings or ignored these results. The opportunity was however taken up in earnest in the West, culminating in the isolation of azadirachtin.

The credit of arousing world-wide interest in the use of neem kernel extract should go to Robert Larson of Vikwood Botanicals, USA, who, during his many business trips to India, had observed the use of neem extractives in rural areas for saving crops from insects and the multifarious uses of the products of neem in villages. With the advice and assistance of USDA, Baltimore, Maryland, USA, he developed a neem kernel formulation, named Margosan-O, standardized to contain 3000 ppm azadirachtin. When diluted 150-fold this would afford a spray solution with 20 ppm azadirachtin, adequate to control many pests. Extensive toxicological studies were carried out on Margosan-O, which was finally cleared for use in horticulture by the EPA in the US.

Very recently, a number of firms in India have begun the production of neem formulations for use in agriculture and have applied for registration with the Central Insecticides Board (CIB). Most of them are based on neem oil or neem bitters with addition of some dispersing agent and are not standardized in terms of azadirachtin content. Neem oil contains only traces of azadirachtin but compounds like salannin, meliantriol, etc. are present which may be useful in the control of some insects. However, formulations based only on neem oil can never match the efficacy of those with the optimal concentration of azadirachtin. Indiscriminate promotion of such formulations could lead to the erosion of faith in the efficacy of neem formulations as a whole.

We in India are favourably placed in regard to the availability of neem kernel for the manufacture of an effective and non-toxic product for use in agriculture. It may not be necessary or possible to eliminate the use of all synthetic pesticides, but neem formulations can in specific cases prove more effective and in general reduce harm to the environment and hazards in agricultural operations. Neem has been subjected to world-wide scrutiny to a degree not accorded to any other plant and a fund of information exists which attests to its usefulness for insect control. Everything should be done by our scientists to exploit this opportunity of providing a safe and effective product for use in agriculture from neem, which should be considered a precious national resource.

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