

Neem Tree (*Azadirachta indica* A. Juss) as Source of Bioinsectides

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1. Introduction

The interest in developing pesticides of natural origins has increased during recent years, because of the drawback of synthetic chemical pesticides, like impact on environment, toxicity to non target organisms including humans, resistance development in insect population. Furthermore, other considerations emerged, including low cost, renewable raw material, like wasting, interest for a possible individual use in urban area. In this paper attention was focused on products of neem tree, *Azadirachta indica* (A. Juss, 1830), and in particular on neem cake, the by-product obtained in the cold-pressed process of neem oil production. Actually neem cake marketed products are not used as pesticides, but mainly as fertilizers. Data on their compositions and insecticidal activity are lacking.

Studies on marketed neem cake products made by HPTLC and HPLC analyses showed differences in their compositions, in particular on limonoids. Once determined limonoid contents in extracts of increasing polarity, these contents were related to larvicidal activity on Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894). The aim of the reported studies is check the possibility of developing a new domestic insecticide using neem cake as raw material, in particular against biting mosquitos present in urban areas.

2. Micro vs. macro competing for life

Microorganisms are difficult to find and to kill, because they are in enormous number and everywhere. For instance, in our body we can count more procariotic than eucariotic cells. Fortunately, most of them are useful friends, but others can be very dangerous and destructives.

Actually, microorganisms are liable of major plagues affecting humans. These invisible our competitors act infections by complicated mechanisms, often involving other creatures. Mosquitos are the favorite partners as major vector of transmission. Therefore, mosquitos are co-responsible of malaria, dengue fever, yellow fever, filariasis, schistosomiasis, Japanese encephalitis, Chagas morbus, hemorrhagic fever, arbovirosis, as well as of several minor pathologies, such as systemic allergic and inflammatory reactions and dolorous bites. Although in developed countries the impact of these pathologies is nowadays restricted or absent, and main causes of death are related to physiological aspects (cancer, hearth and

coronary failures, ecc.), in the remaining predominant part of the world, the alert is always the same and means infection by injure or by bite.

Practically all mankind living in ordinary conditions is continuously exposed to one or more mosquito-borne or connected diseases and suffer in different degrees the effects of the mosquitos attack. Only dengue worldwide threatens the health of around 2.5 billion people, and figures for malaria are surely worst. Malaria infects more than 500 million humans each year. About 90% of cases occur in Africa, including those of malaria-related deaths, but only in India 15 million cases and 20,000 deaths are estimated annually by WHO.

However, as all living beings, also microorganisms have their own Achilles heel. Their movement capacity is very limited, therefore they use animals as vectors for efficiently diffusing in every habitats. Usually, they change to adapt to the host, accumulating therein and becoming vulnerable. Therefore, the option seems to be simple: kill the vector and kill the microorganism.

3. Fighting the vector

Several strategies have been proposed against microorganism/mosquito based diseases in order to control or at least limit mosquitos invasion, mainly based on three types of action: direct, environmental, indirect. Direct methods use as target the adults, whereas indirect methods are mainly focused on effects on mosquito development, including controls of larvae by hormones or other growth regulators. Environmental methods are based on change in the habitat of the insect and display severe collateral effects on other organisms.

So far, mainly synthetic insecticides have been produced and used, in large quantities and types. Initial euphoria for the resolving effects has been punctually followed by negative drawback. Chemical pesticides resulted non-selective, that means harmful and toxic to other organisms including humans, plus the cause of a series of unexpected and durable environmental damages. Several challenges continue to hinder efforts to effectively control vectors, including the induction of insect resistance, insect adaptation and altered behavioral traits, such as exophily and exophagy, not considering limited resources that affect conventional use of control methods in so many countries. The main problem was their inefficacy during the time, leading to the urgent need for novel effective insecticides, rising from natural products.

4. Focusing on larvicides

Generally, interest on biopesticides followed that in natural products applications. Botanical insecticides started in the early 1930s and continued to the 1950s, but it was eclipsed when synthetic insecticides appeared on the scene. However, during the last two decades a revival of natural products overbore in all markets, in coincidence with the difficulties in using synthetic insecticides. Among the important and decisive struggle against microorganism/mosquito based ailments, vector control is actually considered the most feasible way, meeting the modern criteria of use in integrated pest management programs. However, current vector control methods are based on target the adult in order to reduce the vectorial capacity. These control methods must consider behavioral changes of adult mosquitos that can reduce the effect. Nowadays, for these reasons research was focused on larval control, as an overlooked method, better to be used in an integrated vector management program, that means that the environmental care is particularly noteworthy.

However, as well known, indiscriminate methods of applications, larger and unnecessary quantities and concentrations of pesticides are rampant and difficult to limit, often allowing high loads of xenobiotics to reach the soil matrix and accumulate. Therefore, concerns about chemical insecticides and their persistence effects on the environment, as evidenced by the paradigmatic case of DDT (Mulla and Su, 1999), as well as development of physiological resistance by the insects, have stimulated the search of new ecofriendly products. This also in line with 1997 World Health Assembly resolution 50.13, section 2.4 (WHO, 2004; WHO, 2005). The interest in developing bioinsecticides increased dramatically during the recent years, because of the frequent use of synthetic products in urban areas with increasing concern for toxic effects on humans and pets. Furthermore, the invasion in Europe of new more aggressive species was registered. As observed, urban habitat generates in some species effects in terms of increases of pupae production that the "traditional" ones and also complicates the use of chemical insecticides.

Nowadays, attention drove to natural substances produced by plants. A botanical insecticide should be: ecofriendly, biodegradable, target-specific against mosquito vectors. Furthermore, requisites for its market success could be: low cost, availability, easy utilization and simple storage.

As a paradox, the problem consists in the excess of the offer, i.e. the quantity of plants and natural products as possible real candidates. It is estimated that around 100 000 secondary metabolites have been isolated, mainly from plants, but the total number should be much higher, being only angiosperms more than 330 000 species, wherein only the 10% has been the object of relevant phytochemical interest. So far, at least more than 2000 plant species reportedly possess potential for pest control and several are already present in the market. Only in the family of Meliaceae six species have been studied for pesticidal properties (Mulla and Su, 1999) and at least 35 biologically active compounds identified. In any case, random studies are costly and complicated and a strategy of selection must be constructed.

5. Selecting botanical candidates

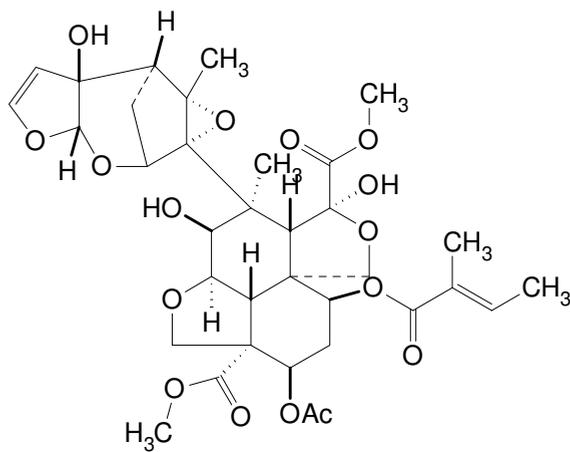
As the history of medicine teaches, information derived from traditional uses can be very useful. Among the plants so far selected as best candidates for developing of natural insecticides, the neem tree, *Azadirachta indica* A. Juss, has already gained a special place, with its reported activity against 400 insect pests. Among the Meliaceae, the Mohogamy family, the genus *Azadirachta* consists of few species, the most important being *A. indica*, a moderate to large tree. It easily and rapidly grows, reaching 80 cm in one year and survives even on dry, nutrient-lean soils (Pundy, 2000). Native to the Indian subcontinent, where is normally found, from Uttar Preadesh to Tamil Nadu, neem continuously spreads in the world. Being a very valuable forestry species, as a multipurpose tree, it is considered ideal for reforestation programs and is largely cultivated world widely, including Central and South America, several countries of Africa and several parts of Asia, like China and Vietnam. Several varieties are also reported, like in Thailand, the *A. indica* var. *sinensis*, locally called the Sadao tree. Limitations concern the preference for tropical and subtropical areas and altitudes not higher than 1000 m. It is often confused with the Indian Lilac, *Melia azedarach*, also known as Chinaberry tree or cinnamon or Santa Barbara in Brazil.

6. Using neem tree

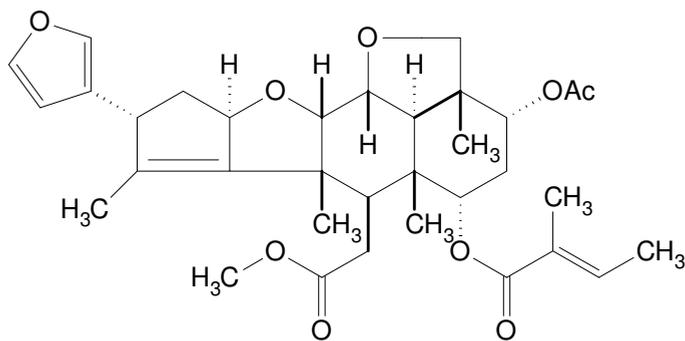
The International Scientific Community, given the enormous amount of results that validate the medicinal properties of Neem, includes neem tree into the top ten list of plants to be studied and used for the sustainable development of the planet and the health of living beings. Neem, identified by WHO/UNEP1989 as an environmentally powerful natural pesticide, is considered to be one of the most promising trees of the 21st century for its great potential in pest management, environment protection and medicine. In this case, sustainability is very high. Not considering that commercial uses are actually restricted the use of removable parts, like leaves, fruits and seeds, there are about 14 million neem trees growing only in India and the plant is adapted to subarid and subhumid areas of tropical and subtropical areas. Indian trees have the potential to produce 3.5 million tonnes of seed/year, corresponding to a production of 700,000 tonnes of oil/year. Neem is well known and used for its medicinal properties from the ancient period (4000 BC); mainly on the indications of Ayurveda medicine, being very popular, even revered in the Indian Subcontinent (Gajalakshimi S. and Abbasi S.A., 2004). In practice, all parts are traditionally used for a variety of indications, but limiting to the ethnobotanical indications concerning the aim of this paper, we can recall the use of neem in indigenous medicine as a bitter tonic, antimalarial, antipyretic, anti-inflammatory, antihelmintic, and for antimicrobial and antiviral effects (Varie, 1996). Fruits are collected when the drupes turn yellowish-green by hands or machines and are processed as soon as possible. The fleshy part of the fruit is removed and the stones washed in clean water and dried for 5-10 days. The oil is obtained usually by large mechanical expellers or by solvent extraction, only small-scale producers still use traditional pressing methods.

The seeds contain about 45% of a brownish-yellow of fixed oil, mainly constituted by oleic acid (50-60%), palmitic acid (15-19%), stearic acid (14-19%), linoleic acid (8-16%) and characterized by an acrid taste and a persistent and unpleasant odour (Mongkholkhajornsilp et al., 2005). The essential oil reported hexadecanoic acid (34.0%), oleic acid (15.7), 5,6-dihydro-2,4,6-triethyl-(4H)-1,3,5-dithiazine (11.7), methyl oleate (3.8), and eudesm-7(11)-enol, as determined by GC-MS (Kurose and Yatagai, 2005). There are also many other constituents reported, i.e. pigments, polisaccharides, salts and the proteinaceous material that makes up the cellular matrix of the seeds (Johnson & Morgan, 1997). However, the quality of the oil is highly affected by the method of processing, as well as the types of seeds. More than 300 compounds have been characterized from neem seeds, with over 50 different bioactive constituents from various parts of neem tree. One-third of reported constituents belong to the class of nortriterpenes, and more precisely to the steroidlike tetranortriterpenoids, named limonoids. Neem limonoids belong to nine basic structures: azadiron (from seed oil), amoorastatin (from fresh leaves), gedunins (from kernels), salannin (from fresh leaves and seeds), and azadirachtin (from seed oil). Azadirachtins (from A up to H), highly oxygenated C-secomeliacinlike compounds, are the predominant and the most studied, including azadirachtin A (AzA), AzB, nimbin, salannin as the most important (Silva et al., 2007 and reference therein), any single main constituent is present with a series of derivatives, i.e. nimbim, nimbinin, nimbidin and nimbidiol. Several marked differences have been reported in the yield of limonoids neem seeds due to geographical origins or even by collection in different seasons in the same area. AzA usually resulted the prevalent constituent followed by AzB (3-tigloylazadirachtol) as second, with concentration up to 15% of the total Az. The different formulations of neem expeller oil have a content of azadirachtin from 300 to 2000 ppm. (<http://www.agroextracts.com/>).

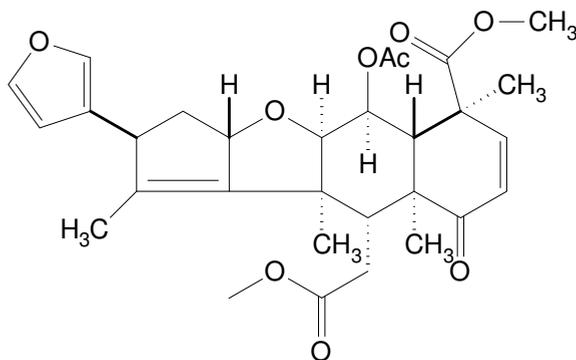
Like most of nortriterpenoids, limonoids are exceedingly bitter. They have attracted global attention for their insecticidal, fungicidal and nematicidal properties (Gajalakshmi and Abbasi, 2004 and references therein).



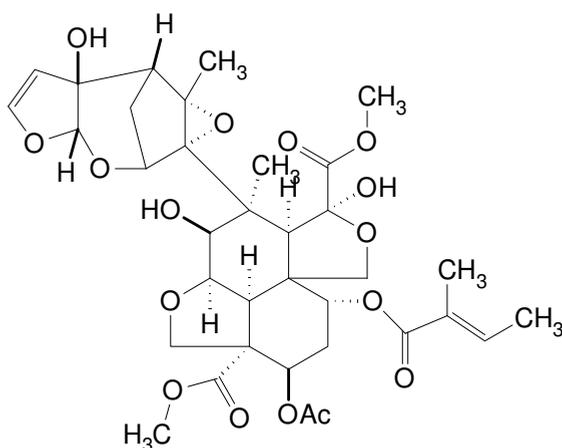
azadirachtin A



salannin



nimbin



azadirachtin B

The insecticidal properties of neem products were first reported by Chopra in 1928. Effects of neem products against mosquitos are well known and documented, both at research level and practical uses. A list of tested arthropods, at different growing stated up to 1993 can be found in paper of Mulla and Su (Mulla and Su, 1999). In particular, it protects against the bite of *Anopheles*, it repels *Culex quinquefasciatus* (Su and Mulla, 1999) and *Aedes* spp., it causes nymphal death in *Bamisia tabaci*. Larvicidal action was reported on the dengue mosquito *Aedes aegypti* (Ndione et al., 2007), the malaria vector, *Anopheles gambiae* (Okumu F.O. et al., 2007), and the filarial vector, *Culex quinquefasciatus*. These actions are based on multiactions against insects: toxicity, antimittotic effects, antifeedant activity, growth regulation, fecundity suppression, sterilization, oviposition repellency, including harmful effects on endocrinien system and damages of the cuticle of larvae preventing them from moulting (Mulla and Su, 1999). Also aqueous extracts of neem wood and bark chippings showed larvicidal activity against *A. gambiae* (Howard, 2009); in this extracts HPLC analysis

showed the presence of a series of constituents of varying polarity, including nimbin and salannin, whereas AzA was not detected. Structure complexity of Az and other bioactive neem constituents precludes any large-scale chemical synthesis by the extremely high cost. Therefore, the only practical option is the extraction from renewable parts of the tree, i.e. leaves and seeds, and manufacture various pesticidal formulations.

Neem preparations that resulted effective against insecticide-resistant pests, yet does not harm heavily the beneficial insects. Out of date, neem products are considered relatively safe to mammals and humans (Boeke et al., 2004). Anyway, the absence of toxicity is largely tested by the prominent role of fruits, bark and leaves in Indian Traditional Medicine, as well as the use of flowering and leaves as vegetables in Asian countries and the current use in a number of toiletry and pharmaceutical products. Acute toxicity of neem oil has been studied and is reported as 12 ml/Kg for rats and 24 ml/Kg for rabbits (Gandhi, 1988). General cautions must be taken for the possible presence of aflatoxins, usually present in case of bad storage, as in other botanical raw materials.

The results showed that neem products could act primarily as larvicides (Zebitz, 1986, 1987; Naqvi et al., 1991; Rao et al., 1992, 1995; Amorose, 1995; Wandscheer et al., 2004; Okumu et al., 2007, Howard et al., 2009). Organic solvent extracts and oils from neem and *Melia azedarach* have displayed several bioactivities against insects governing chemical maturation of molt hormones, chitin biosynthesis and field deterrence. Activities were referred to limonoids, i.e. Az from neem and meliacarpinin from *M. azedarach*.

7. Using neem cake

Procedure for obtaining neem cake oiled and deoiled is summarized in Fig.1. However, the extraction processes are subjected to several changes, depending the producer.

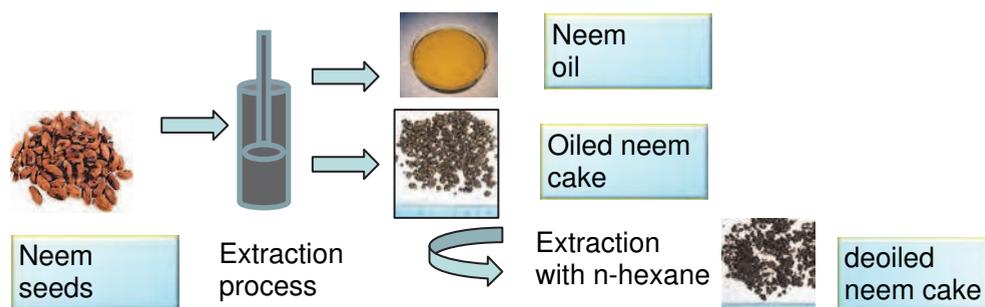


Fig. 1. The principal steps leading to the production of deoiled neem cake.

The commercialization of neem cake, usually from India, in the European market is already a reality as is allowed its use as fertilizer in organic farming and organic livestock feed supplement, in accordance with current legislation. For now, the control of product quality, only provides a label stating the contents of N, K, P and information on the type of extraction processes by which the cake comes (cold-pressed or cold-pressed, followed by extraction with organic solvent). The purpose of this study is focused on demonstrating how

the technology HPTLC can be as useful for the labeling of the product. The neem oil cake is the by-product obtained in the cold-pressed process has an oil content of about 6%, while the neem deoiled cake, the by-product obtained in the organic solvent extraction process, has still an oil content of about 1.5 %. The only present utilization of neem cake concerns its use as a natural and environmental friendly fertilizer, soil conditioner, nitrogen saver and manure in farming and agriculture (Gopal et al., 2007). Deoiled neem cake is traditionally used as fertilizer by Indian farmers, which appreciate also the pest repellent effects. When neem cakes is ploughed into the soil, it also protects plant roots from nematodes and white ants. The ethnobotanical indications were fully confirmed by recent studies, showing that neem cake at the same time acts as a pesticide and provides the much necessary nutrition to the soil microbes, besides improving the soil physico-chemical properties.

Neem cake seems to be a good pretender for developing new botanical insecticides. Beside the already mentioned qualities, we must stress on the global interest in recycle and intelligent utilization of materials considered a waste or exhausted. Moreover, it is also clear that such projects need a lot of technology, research and initial funding, but sometimes results pay the expectative of long years of studies and tentatives. For instance, a constant increase of potential yield of main crops is urgently requested, calling for massive quantities of fertilizers and pesticides. Appropriate technologies, including the use of low cost natural products should have key role to environmental safeguard. Another crucial aspect concerns the use of neem cake as agro-industrial by-product for livestock feed, as several researches and applications have suggested (Verna et al., 1995; Rao et al., 2003). Neem-cake is currently used as organic fertilizer and as feed supplement in animal husbandry. India alone has an annual potential of 80,000 tons of oil and 330,000 tonnes of neem-cake from 14 million plants that grow naturally. Neem cake is therefore a product of low cost and widely available on the world market. In particular, starting from the neem cake has been isolated a phytoextract that has a remarkably high insecticidal activity against larvae of *Aedes albopictus*, commonly known as tiger mosquitoes (Nicoletti 2010).

8. Looking for the ideal insecticide

The raw material to be used for a domestic insecticide should be:

- low cost and abundant
- no toxic itself or by processing except to the target
- composition must be determined as deep as possible and the used product stable in determined composition
- biological activity reported in details and tailored for target organism.

Our research was focused on the last two items, and *Aedes albopictus* selected a target organism owing its increasing negative role in Europe, and in particular in Italy. In the last decades, the spreading of Asian tiger mosquito, all around the world, has caused mainly the colonization of towns environment. This blood sucking mosquito raised serious concern, because its bites cause great trouble and it is a competent vector for the transmission of at least 22 arbovirus (Gratz, 2004). As a matter of fact, Asian tiger mosquito was absent in Italy since two decades ago, but after its introduction it rapidly became dominant, leaving other competitors to a secondary role. So far, adopted methods by Municipalities resulted deprived of real efficacy. Local transmission of the *Alphavirus chikungunya* (CHIK) has been referred in two small towns in the province of Ravenna,

Italy during the 2007 summer (Vazeille et al., 2007) At the present, the most spread chemical insecticides used as mosquitoes larvicidae includes organophosphates, pyrethroids and insect growth regulators. In spite of the increasing of pollution by residue of synthetic pesticide in towns environment, this is an inevitable threat to citizen health, but the lack of mosquito management in private areas makes the level of infestation by *A. albopictus* out of control. According to the insect biology, one of the most important reproduction sites chosen by the insect are saucers flower pots and road drains, requiring a domestic and capillary use of the insecticide. Therefore, pesticide effects must be tested and performed in aqueous conditions.

Being biodegradable by action of sun light, neem products do not leave any residue on the field. The degradation of Az under field conditions is quite rapid and takes place by the effects of UV light, temperature, pH and microbial activity, avoiding accumulation. However, neem commercial success has been limited by the relatively high cost of the refined product and the low persistence of azadirachtin activity on crops exposed at sun light (Isman, 2006). These two aspects lead to explore the production of new neem products maintaining the insecticidal activity and helping the commercialization.

9. Analyzing neem cake products in four steps

The exact determination of neem products chemical composition can be considered the Mont Everest of the analytical study. Besides the aforementioned constituents of the neem oil, already object of several studies, like limonoids and fatty acids, determination of composition of neem cake composition is a hard, but necessary work. Apparently, this complexity could be of great importance for the larvicidal activity of neem cake, since minor compounds could be important copartecipants of the activity, being crucial in solubility and biodisponibility in accordance with the phytocomplex phylosophy. To face the complexity of neem cakes composition two analytical approaches were used: HPTLC was selected to evidence the total spread of constituents and HPLC to define quantities of limonoids.

High Performance Thin Layer Chromatography - HPTLC - is the last frontier of planar chromatography, becoming one of the best methods for control of quality, purity, stability and identity, in one word validation of complex botanical products (Reich, E. & Schibli, A. 2007). Besides the achieved great increasing in efficacy, due to the use of minor size silica gel that means larger surface, it is possible to perform high quality HPTLC analytical determinations, due to novel dedicated machinery, in order to finally achieve the necessary reliability, repeatability and flexibility. Substantially, the advantages of HPTLC are consistent with the rapidity and the possibility of analysing many samples at the same time under the same chromatographic conditions. The same HPTLC plate can be visualised with and without derivatisation using different light sources, obtaining an enormous quantity of information. Comparison is easy by the fingerprint approach. A fingerprint is the individual chromatographic track representing, as near as possible, the mixture of produced organic substances. By the fingerprint approach, it is possible to obtain a proper identification of the plant material, but also determine and assert the limits of the biological changes, without necessary identifying nor quantifying a specific compound(s). In HPTLC tracks of the same species variations are mainly quantitative, not qualitative. The fingerprint approach is very useful in the analysis of complex mixtures. HPTLC results a simple, rapid and useful method to obtain a general and almost complete information about the composition of neem cake products. Many products can be compared side-by-side with standards and

densitometry allows a quantitative analysis, opening planar chromatography to the 3D dimension. The fingerprint approach was introduced and accepted by WHO, as useful analytical technology for identification and quality validation of herbal products (WHO, 1991). The possibility of a correct use in estimation of AzA, AzB, salannin and nimbin in herbal extracts was demonstrated (Agrawal et al., 2005).

The study of neem cake products, based on determination of compositions and relative activities, was performed on four steps.

9.1 First step

HPTLC fingerprints of several neem cake marketed products on comparison with limonoids standards. Beside the expected differences between oil and deoiled, the HPTLC showed great variations in compositions. The Fig. 2 and 3 report a typical HPTLC fingerprint analysis on some of marketed neem products obtained by the collaboration of producers or importers; the plate is the same, but in Fig. 2 the tracks were visualized at the white light after derivatisation with anisaldehyde, whereas in Fig 3 the visualization at 366 nm was used, in order to obtain further information on fluorescent compounds. The plate starts with tracks 1-6 where same limonoid standards were reported. It is noteworthy that several standards appear not pure. This is an effect of the extreme sensibility of HPTLC. In fact, although the same standards appeared sufficiently pure at the NMR inspection, HPTLC is more inspective. Track 7 reports the total fingerprints of utilized limonoid standards. Track 8 is dedicated to the analysis of a sample of neem oil directly obtained from India. The fingerprint shows a presence of limonoids, but with salannin and nimbin as the most evident limonoids whereas AzA results a secondary components, instead of the most reported analytical data. It is also evident the presence of the oil components well evident as a strong spot near to the front of the plate. The HPTLC analyses of commercial neem cakes obtained from different producers, importers and markers revealed great differences in the compositions. As expected on the basis of deoiling process, great differences concerned the quantity of fatty compounds. These compounds can be evidenced as strong bands of red shining color at 366 nm, whose identity was confirmed by isolation by CC in *n*-hexane/ethylacetate 9:1 and identification by ^1H and ^{13}C NMR in CDCl_3 .

Tracks 9-15 are dedicated to neem cakes, using methanol extracts to obtain the most complete extraction: in general, fingerprints are very similar, confirming positively the neem origin, as well as differences can be observed in the intensity or presence of single spots. Track 9 presents the methanol extract of the previous product of track 8. A second strong band can be now observed near the middle of the fingerprint, that was assigned to mixtures of alcohol and methylester derivatives of the former fatty acids by NMR analysis. It is evident that the presence and/or predominance of fatty constituents can have great influence in the physico-chemical properties, as well as activities, of the different neem cake products. The same spot is also evident in tracks 10 and 11, but not so present in the other fingerprints. The contents of limonoids also vary in each fingerprint, as better evidenced in Fig. 3. Focusing on the presence of salannin, its occurrence appears in all the products, although at glance the quantity can not be derived. Situation for nimbin and AzA is completely different.

Attention was focused on the product of track 14, containing limonoids, but also great quantity of fatty constituents. Therefore, we developed a method to clean the ethylacetate

extract from most of the fatty compounds first by extraction of the total extract with ethylacetate, followed by precipitation in ethylether/ethylacetate solution, obtaining as evident by the consequent fingerprint of track 16, two main results: a) differences in the presence of limonoids were better appreciated and solubility in polar solvent increased; b) it was evidenced the presence of a series of compounds strongly fluorescent at 366 nm light, previously obscured by the fatty band. Further studies are in progress to assign the definitive structures of these substances.

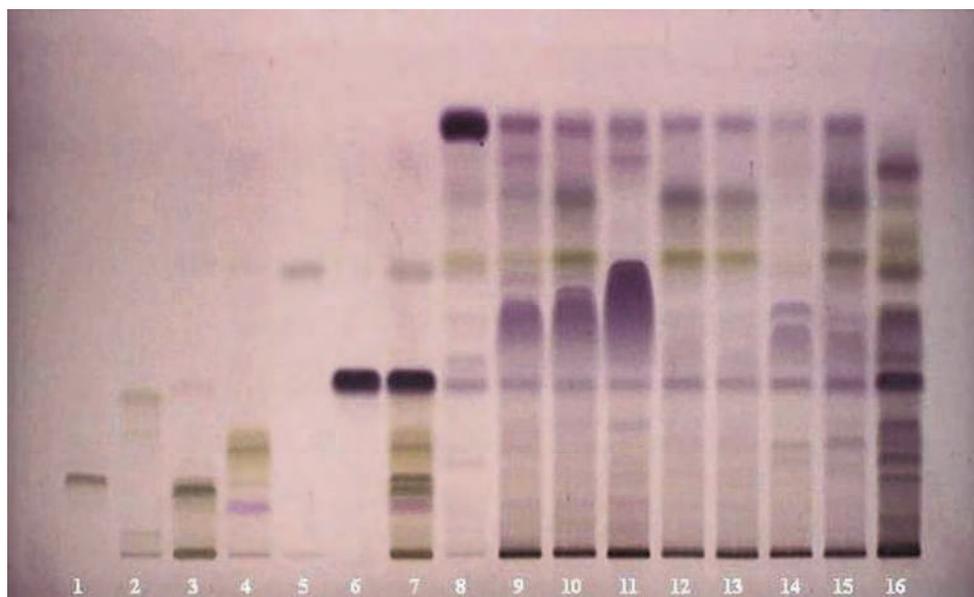


Fig. 2. HPTLC fingerprints of neem products and selected standards. Mobile phase: toluene, ethyl acetate (4:6). Derivatisation: Anisaldehyde. Visualization: white light. Tracks: **1** azadirachtin A, **2** azadirachtin B, **3** azadirachtin D (11-epi-azadirachtin A), **4** 11-deoxy-azadirachtin A, **5** nimbin, **6** salannin, **7** the previous standards all together, **8** neem oil marketed in India, **9** methanol extract of neem cake from India, **10-15** methanol extract of commercial samples of neem cakes; **16** neem cake of sample 14 re-extracted with ethyl acetate.

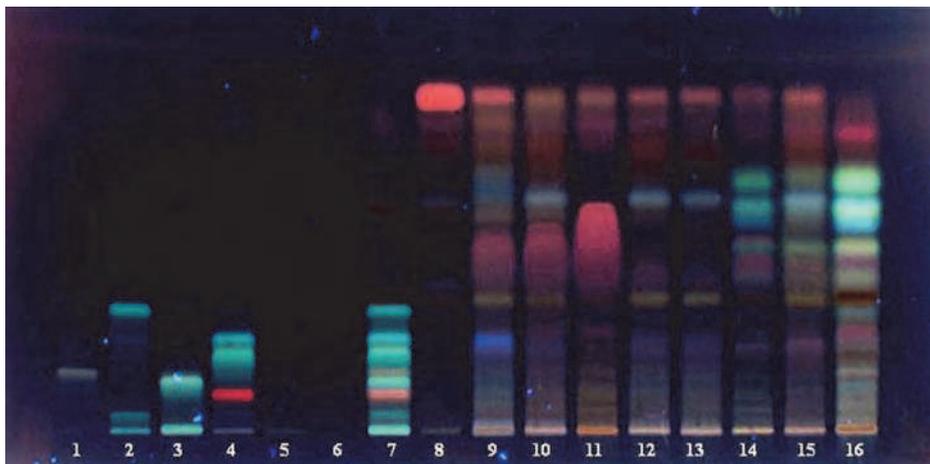


Fig. 3. The same tracks of Fig. 2 visualized at UV366 nm. In particular, by separation and NMR identification the main spots evident in the middle of the tracks 10-12 resulted as a mixture of fatty constituents.

9.2 Second step

Analysis of neem cake by HPLC. In order to better analyse the differences in neem cakes, neem cake of track 14, which was selected as test product for its complexity, was analyzed by HPLC for content in limonoids. Fig 4 shows the typical HPLC chromatogram of the methanol extract of neem cake, performed in gradient of water/CH₃CN 30-60% using a LC-18 column. The analysis, performed to evidence the nortriterpene presence, showed the following results: AzA (2750 ± 100 ppm), AzB (1000 ± 15 ppm), salannin (7980 ± 50 ppm), nimbin (1850 ± 100 ppm) (Nicoletti, 2010). Therefore, also after the industrial treatment of extraction, neem cake still contains relevant quantities of nortriterpenes.

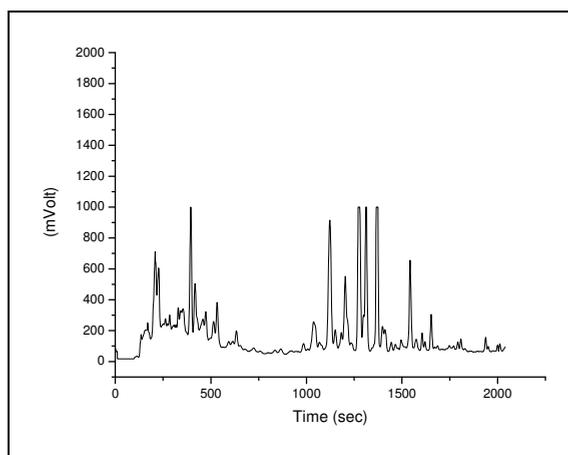


Fig. 3. Typical HPLC chromatogram of methanolic extracts of a neem cake products evidencing the limonoids in the middle of the chromatogram.

The main obtained evidences in composition analysis can be now summarized:

- composition of neem cakes is different from that of neem oils.
- differences in general composition of the marketed neem products were evident. The used repartition method resulted not efficient in separating the different constituents. The presence of limonoids in all extracts could be evidenced, although in different amounts as determined by HPLC. Thus, for instance, salannin resulted the most predominant limonoid, with low quantities of AzA. This result is in contrast with analysis of neem oil.
- HPLC showed a precise figure of the limonoid contents, allowing a comparison between these data and larvicidal activities on *Aedes albopictus*.
- constituents of different chemical structures from those already reported can be accumulated during processing steps.

9.3 Third step

Test of the larvicidal activity of different extracts. Therefore, the dried total extract was washed with *n*-hexane and then dissolved in a water/ethylacetate mixture, and the resulting water phase reextracted with *n*-butanol, in order to obtain four extracts partially concentrated in constituents of increasing polarity (named Hp for the *n*-hexane, Ep for the ethylacetate, Bp for the *n*-butanol and Wp for the water).

Larvicidal activities of the four extracts (Ep, Hp, Bp and Wp) of neem cake product of track 14 was determined, in order to locate presence and polarity of active principles. The test used *Aedes albopictus* a week old eggs, still laying on their paper substrate, after one day of drying, when submerged in the tested solutions, hatch. The results show that ovidal activity doesn't occur when a week old eggs are deposited in neem derivatives. Tables 1, 2, 3 show that the percentage of hatching occurs without significant differences in all tested solutions in respect of the control.

The new born larvae were allowed to develop in the tested solutions and the larval mortality was assessed after 2-4-6-8-days. At day 8, Ep and Hp (Table 1) show a high larval mortality compared to control, to Bp and to Wp. In table 2 the Ep activity is compared to that of different concentration of AzA: at the highest AzA concentration, Ep present a higher mortality after 8 days. At day 8, the Ep (Table 3) shows the same effect, in terms of larval mortality, of the Dirachtin solutions at Az 10-100-1000 ppm.

Samples	Mean hatch (% ± DS) ¹	Mean mortality (% ± DS) ¹ after 2 and 8 days	
		2	8
control (H ₂ O)	63.33 ± 21.86 ^a	6.90 ± 6.20 ^a	6.90 ± 6.20 ^a
Bp	53.33 ± 5.77 ^a	6.67 ± 11.55 ^a	6.67 ± 11.55 ^a
Wp	43.33 ± 14.53 ^a	2.22 ± 3.85 ^a	15.56 ± 26.94 ^a
Ep	36.67 ± 14.53 ^a	4.95 ± 4.29 ^a	86.94 ± 5.17 ^b
Hp	35.56 ± 18.95 ^a	3.70 ± 6.42 ^a	88.78 ± 5.39 ^b

¹ Different letters in horizontal line indicate significant differences in hatching and mortality rate of larvae by Tukey's Test at 0,05 level.

Table 1. Comparison of the activity of various neem-cake extracts against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days (Tukey's Test).

Samples	Mean hatch (% ± DS) ¹	Mean mortality (% ± DS) ¹ after 2 and 8 days	
		2	8
control (H ₂ O)	63.33 ± 21.86 ^a	6.90 ± 6.20 ^a	6.90 ± 6.20 ^a
Az_A 0.1 ppm	44.44 ± 1.92 ^a	2.56 ± 4.44 ^a	10.26 ± 17.76 ^a
Az_A 0.5 ppm	55.33 ± 14.53 ^a	3.17 ± 5.50 ^a	3.17 ± 5.50 ^a
Az_A 1.0 ppm	47.78 ± 15.75 ^a	0,0 ± 0,0 ^a	5.79 ± 5.57 ^a
Az_A 5.0 ppm	54.44 ± 5.09 ^a	0.0 ± 0.0 ^a	27.69 ± 18.22 ^a
Az_A 10.0 ppm	48.89 ± 6.94 ^a	13.21 ± 5.06 ^b	80,49 ± 10,99 ^b
Ep	36.67 ± 14.53 ^a	4.95 ± 4.29 ^a	86.94 ± 5.17 ^b

¹ Different letters in horizontal line indicate significant differences in hatching and mortality rate of larvae by Tukey's Test at 0.05 level.

Table 2. Comparison of the activity of Ep and technical azadiracthin solutions at various Az_A concentrations against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days. (Tukey's Test)

Samples	Mean hatch (%±DS) ¹	Mean mortality(%±DS) ¹ after 2 and 8 days	
		2	8
control (H ₂ O)	28.89 ± 5.09 ^a	3.70 ± 6.42 ^a	14.44 ± 17.11 ^a
Dirachtin(Az 1.0ppm)	24.44 ± 5.09 ^a	31.75 ± 2.75 ^a	36.51 ± 5.50 ^a
Dirachtin(Az 10.0ppm)	31.11 ± 1.92 ^a	22.22 ± 22.22 ^a	85.56 ± 6.76 ^b
Dirachtin(Az 100ppm)	27.78 ± 1.92 ^a	51.85 ± 37.64 ^{a,b}	92.13 ± 6.85 ^b
Dirachtin(Az 1000ppm)	30.0 ± 3.33 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b
Ep	30.42 ± 12.73 ^a	3.75 ± 3.89 ^a	84.34 ± 4.65 ^b

¹)Different letters in horizontal line indicate significant differences in mortality rate of larvae by Tukey's Test at 0.05 level.

Table 3. Comparison of the activity of Ep and Az commercial formulation at various concentrations against *Aedes albopictus* as indicated by the hatching rates of eggs and larval mortality after 2 and 8 days. (Tukey's Test)

9.4 Forth step

Evaluation of larvicidal activities of neem cakes aqueous solution, obtained by soaking 3,7 g in 100ml of raining water, in relation with limonoids contents. The previous studies can be now combined to obtain a final result. The HPTLC analysis of the six selected marketed neem products was performed to properly evidence differences in compositions (Fig. 4).

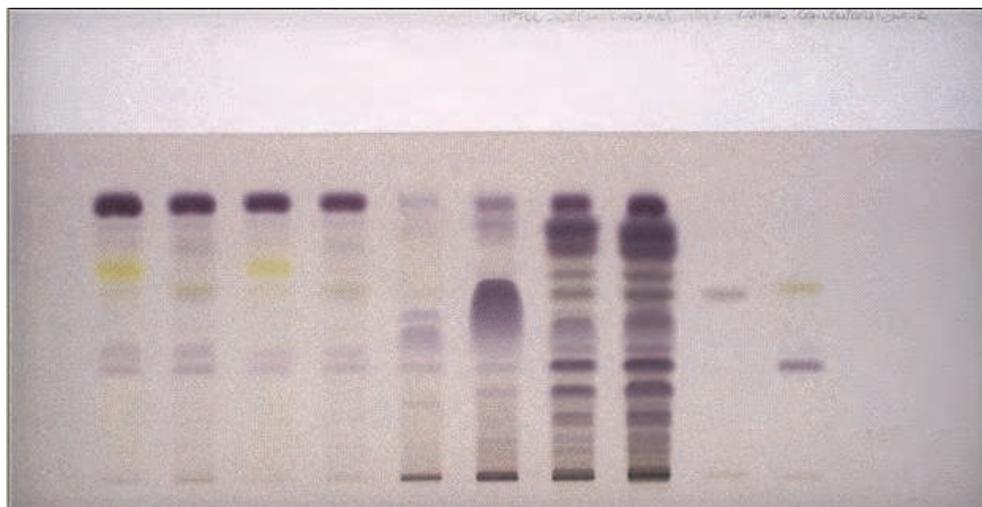


Fig. 4. HPTLC analysis of selected neem cakes. Tracks 1-6 fingerprints of six marketed neem cakes; tracks 7-8 neem cake of track 6 reextracted with ethylacetate and defatted in two concentrations; track 9 nimbin; track 10 salannin.

HPLC analysis confirmed the prevalence of salannin, whose quantity changes greatly in the different products. The larvicidal effects evidence a good correspondence between salannin content and larvicidal activity.

Neem cake	AzA (ppm)	Nimbin	Salannin
1	79	26	858
2	15	84	266
3	107	117	190
4	185	126	1260
5	184	321	2700
6	40	250	1390

Table 4. HPLC results on quantitative determination of main limonoids in neem cake marketed products.

Neem cake	2 days	3 days	4 days
1	2.2 ± 3.8	94.1 ± 10.2	97.9 ± 3.6
2	50 ± 50	50 ± 50	70.0 ± 30
3	3.0 ± 5.2	3.0 ± 5.2	3.0 ± 5.2
4	93.3 ± 5.9	93.3 ± 5.9	93.3 ± 5.9
5	86.7 ± 23.1	100.0 ± 0.0	100 ± 0
6	65.3 ± 17.7	90.2 ± 9.2	100 ± 0
Control	22.6 ± 20.9	22.6 ± 20.9	22.6 ± 20.9

Table 5. Larvicidal effects of different neem cakes on *A. aldopictus*

10. Concluding

The complexity of neem cake requires a multidevice approach, in order to obtain a great quantity of data in accordance with the different types of natural products present. Total information derives from the complementary use: HPTLC for general composition, NMR for structural determination, HPLC for quantitative determination.

The activity observed is mainly preliminary and must be confirmed with the study on the physiological effects on larvae. A comparison of the results reported with the outcome from other studies concerning the neem products is not easy. Differences can be attributed to the origin of the products, concentrations of active ingredients, the target mosquito, modes of application. However, larvicidal activity of neem cakes on Asiatic Tiger was evidenced. Activity is in some way related to salannin and/or limonoids contents, but the co-operative influence of other constituents must be considered.

The enormous quantity of different compounds allows the possibility of increase the presence of selected constituents by chemical treatment as evidenced in the fingerprint of track 16.

Future steps in the validation of neem cake as possible raw material for the development of a new domestic insecticide will be the study of the physiological effects on larvae, the exact determination of the composition of the most active neem cake products and the development of a solution containing most of the active products and to be used in aqueous environment, like puddles, small marshes, saucers, gutters.

In 1992 U.S. Academy of Science published a report entitled prophetically "Neem, a tree for solving global problem".

11. Acknowledgements

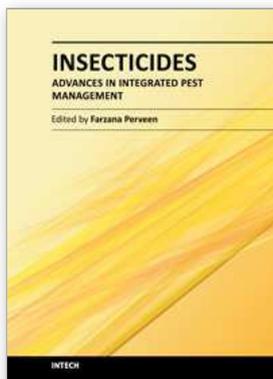
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This book contains 30 Chapters divided into 5 Sections. Section A covers integrated pest management, alternative insect control strategies, ecological impact of insecticides as well as pesticides and drugs of forensic interest. Section B is dedicated to chemical control and health risks, applications for insecticides, metabolism of pesticides by human cytochrome p450, etc. Section C provides biochemical analyses of action of chlorfluazuron, pest control effects on seed yield, chemical ecology, quality control, development of ideal insecticide, insecticide resistance, etc. Section D reviews current analytical methods, electroanalysis of insecticides, insecticide activity and secondary metabolites. Section E provides data contributing to better understanding of biological control through *Bacillus sphaericus* and *B. thuringiensis*, entomopathogenic nematodes insecticides, vector-borne disease, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

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