CHAPTER SEVEN

New solutions using natural products

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Dr. Bruce Reid in his *Principles of Botany* wrote: "Genes determine what an organism can do, the environment determines which of the things that an organism can do the organism will do. A given species may be constructed differently in different environments." Therefore, genes = can, environment = will. Natural products are the bridge between the environmental wellspring of experience and the yearning for changing the world in one's own favor. These products are the result of the combined effects of the possibilities and limits of the genome and the environmental inputs. Each organism, as well as creating its best and adequate form, synthetizes its chemical arsenal, necessary to realize and consolidate its individuality inside the habitat. This arsenal is mainly dedicated to the interactions with abiotic

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and biotic targets, which are important to the individual homeostasis and survival. One form of evidence in accordance with this interpretation is the strong correlation between natural products' producers and biodiversity, therefore indicating a matter of adaptation.

However, it is possible to argue that if differences can characterize each organism from another, the some is not true in case of a molecule. Once determined the structure, a molecule is a molecule, even if generated by the metabolism or by a synthetic route, and therefore the living rules cannot be applied to the molecular world. This is true only in part. We must consider, in particular for natural products, the conformational forms, which are different ways of the same molecule to react. An organic molecule can change easily its 3D structure adapting to the environmental needs, like we open and close our hands. Furthermore, once inside the metabolism, the molecule, even if inorganic, is integrated in the organic network and it is forced to collaborate to an integrated living system. The cell environment is far different from the test tube of the chemist. In practice, the molecule is simply part of an advanced complex integrated dynamic system, limiting its freedom. Beside the consideration that physical forces work in the same way in the assembling matter, as we can know and distinguish a giraffe from a wizard, we can study natural compounds and distinguish them on basis of the structures and assign their place and role. About the role, we must remember that natural products are chemical mediators inside the environment, meaning that they must act on the receptor of the target organisms. Therefore, the structures of natural products are derived from their activity, the same argument we use in the consideration of a pharmacological drug. Here, the importance of natural products inspiring all the molecules tailored to affect living organisms. In particular, among the detected activities in plant species, the defensive against herbivorous is highly reported. Plants cannot counteract by movements like animals and therefore an arsenal of chemical weapons is essential in their fight for survival. Therefore, it is highly possible to find natural products toxic or repellent to phytophagous, like insects, as well as active constituents against pathogens. Natural products with such properties should be extracted and found in the complex and confused reservoir of secondary metabolites, but the presence into the plant is not sufficient. It is necessary to realize the mechanism of action and the potentiality to be used as marketed products. Therefore, the pathway should comprehend the discovery, the isolation, the sources, the bioactivities, and the possibility to be obtained in high quantity, the method of utilization, the environmental impact and the cost of production.

Essential oils as natural insecticides

The natural products have been already reported for antibiotic activity. In particular, essential oils and phenols are stated in many papers as being responsible of antibacterial and insecticide properties (Ghosh et al., 2012; Gibbons, 2008). However, the activity seems to be too general. Most essential oils are composed of the same main constituents, the difference being mainly quantitative for each compound or hidden deeply inside the plethora of secondary constituents. Furthermore, excluding the phenols typical of essential oils, the quantity of known phenols is very large and complicated, with thousands of structures reported and different activities connected. Therefore, special research must be performed, including the possibility to test new substances never reported.

Among secondary natural products with insecticide activity, a special place must be assigned to essential oils. In this regard, several papers report the utilization of essential oils as the active ingredient against pests. The antimicrobial activity of essential oils is also well-reported and evident in nature. Several plants accumulate essential oils in the inner parts of roots and rhizomes, in order to avoid the devastating attack of micropathogens, wherein the plant often accumulates precious reserve substances. In other cases, the aerial parts are focused on defensive or cooperative actions. Some birds defend their clutch by surrounding the nest with aromatic plants. The human utilization of essential oils of different kinds against insects has a long story. Herodotus reported the use in ancient Egypt of mosquito nets and towers impregnated with fish odor to avoid mosquito bites.

An essential oil is a complex chemical mixture of substances volatile at ordinary temperature (Figs. 7.1 and 7.2), and therefore the constituents must have low molecular weight. In other words, they are micromolecules, with average molecular weight of 120–160 uma and hydrocarbon prevalence. Essential oils can be extracted from the raw materials by utilizing their volatile properties, such as in the steam distillation method. The antimicrobial activity of essential oils is usually a consequence of the content of phenols, but other properties must be considered. On the basis of the structures of the active constituents, there are two types of essential oils. The first one, mainly present in less advanced Angiosperm dicotyledons, like Magnolidae, contains mainly root and fruit drugs rich in simple phenolic phenylpropanoids, which are mainly utilized by the plant in protection of pathogens. In Rosidae and Sympetalae, the terpenoids progressively

Fig. 7.1 A typical current apparatus for the production of essential oils by steam distillation.

become predominant. The new volatile constituents, in addition to the protective and toxic effects, afford a positive attraction on pollinatory agents, evidencing the plant position and allowing a memory of the selected species. In this way, the scenario of the interaction between animals and plants changes from defensive to collaborative. The new plants to be selected and appreciated can enrich the offer to the collaborative animals

Fig. 7.2 A historical apparatus for the production of essential oils. Also shown is the typical mask once used for centuries in Europe in the case of plague.

with fruits of inebriant flavors, colored flowers and other nice experiences. Therefore, utilized essential oils are mainly complex mixtures of volatile plant secondary metabolism and consist mainly of monoterpenes and sesquiterpenes, which means lipid secondary metabolites, and, to a lesser extent, of aromatic compounds. The choice of essential oil depends firstly on the taxonomy of the selected plant and the effects depend on the nature of the constituents of the essential oil. The conclusion, based also on direct experiments, is the presence of a general antibiotic and insecticide activity; however, another real need is a selective toxicity in favor of the useful organisms. Therefore, some kind of activity is expected for an essential oil, but there is a necessity to maximize its effectiveness. They are exploited in several fields, such as perfumery, food, pharmaceutics, and cosmetics, but essential oils have also long-standing uses in the treatment of infectious diseases and parasitosis in humans and animals.

Essential oils, currently more than 300 of which are known, are highly variable in their complex composition. Usually, at least a mixture of more than main 30 different constituents of low molecular weight is present. Among the single species, the qualitative composition of the essential oil is respected, although a quantitative variability is common between population according to the environmental pressures. However, some terpenes can be easily found, like the hydrocarbons (myrcene, pinene, terpinene, limonene, cymene, α- and β-phellandrene) and the oxygenated ones, like the alcohols (geraniol, linalool, menthol, terpineol, borneol), the aldehydes (citral, citronellal), ketones (menthone, pulegone, carvone), bicyclic monoterpene ketones (thujone, verbenone, fenchone), acids (citronellic acid, cinnamic acid), oxides (1,8-cineole) and esters (linalyl acetate), but the aromatic phenols (carvacrol, thymol, safrole, eugenol) are also common. A few essential oils may also contain sulfur-containing constituents, methyl anthranilate, coumarins, and special sesquiterpenes such as zingiberene, curcumin, farnesol, sesquiphellandrene, turmerone, nerolidol, etc. Often these components are at low concentrations (less than 1% each), but the opposite is also possible, with major compounds that can represent up to 70% of the total volume of oil, as much as 90% like eucalyptol in *Eucalyptus* or limonene in *Citrus* or pinenes in turpentine of *Pinus*. Therefore, the antiparasitic activity of an essential oil can vary according to differences in its chemical composition, but it is usually present. Nowadays, there is an increasing interest in the utilization of essential oils against endoparasites and ectoparasites of animals and humans, in particular when they are resistant to conventional drugs. However, the use of essential oils is in general restricted for the high cost and considering that usually they are not adequately specified for the considered target (Bagavan and Rahuman, 2010; Shaalan et al., 2005; Tikar et al., 2018). Again, also in the case of essential oils, the insurgence of the insecticide resistance must be considered (Brown, 1986), although usually less common in these cases. Therefore, in consideration of their general but also weaker effectiveness of essential oils in comparison with synthetic insecticides, their utilization requires the insecticidal properties of essential oils to be investigated in different approaches of selection of the studied plants and their uses. Some examples of researches, in which I had occasion to participate, involving essential oils in insect-borne diseases are reported here. The leading idea was to utilize the essential oil properties in an innovative way, such as in mixture or selected types.

In 2017 (Benelli et al., 2017a,b), the activities of five essential oils were investigated. The essential oils were obtained from different plants: *Pinus* *nigra* var. *italica* (Pinaceae), *Hyssopus officinalis* subsp. *aristatus* (Lamiaceae), *Satureja montana* subsp. *montana* (Lamiaceae), *Aloysia citriodora* (Verbenaceae), and *Pelargonium graveolens* (Geraniaceae) against *Culex quinquefasciatus* (Diptera: Culicidae), which is a vector of lymphatic filariasis and of dangerous arboviral diseases, such as West Nile and St. Louis encephalitis. The research was original in its focus on the potential synergistic and antagonistic effects, testing them in binary mixtures on *C. quinquefasciatus* larvae. Mixtures of essential oils are very easy to obtain, since the constituents are perfectly soluble in the final solution and the selected oils were cheap and easy to find on the market. In such a way, knowing the composition, it is possible to combine constituents, enhancing the range and the quality of activity. The pool of the investigated species was highly varied, but this was considered a positive factor. First, the chemical composition of each essential oil was investigated by GC-MS analysis, which is the best analytic method in such mixtures of volatile compounds. Therefore, it was also necessary to test the activity of each essential oil and later to try the best combination on the basis of its effectiveness. The highest effectiveness was obtained by *S. montana* subsp. *montana* essential oil ($LC_{50}=25.6\,\mu L\,L^{-1}$), followed by *P. nigra* var. *italica* ($LC_{50}=49.8\,\mu L\,L^{-1}$), and *A. citriodora* ($LC_{50}=65.6\,\mu L\,L^{-1}$). It was possible to obtain an enhancement of the larvicidal activity by preparing simple binary mixtures of essential oils (ratio 1:1), such as *S. montana*+*A. citriodora*, which showed higher larvicidal toxicity ($LC_{50}=18.3\,\mu L\,L^{-1}$). On the other hand, testing *S. montana*+*P. nigra* (1:1), an antagonistic effect was detected, leading to an LC_{50} ($72.5\,\mu L\,L^{-1}$) higher than the LC_{50} values calculated for the two oils tested separately. Therefore, these results indicate the extreme need for innovation and imagination in natural products research, against many papers repeating the same procedure that change only the plant used.

Another work (Pavela et al., 2016a,b) was based on geographic distribution and the traditional use. Six medicinal and aromatic plants—*Azadirachta indica* (see later in this chapter), *Aframomum melegueta*, *Aframomum daniellii*, *Clausena anisata*, *Dichrostachys cinerea*, and *Echinops giganteus*—have been traditionally used in Cameroon to treat several disorders, including infections and parasitic diseases. The aim was to evaluate the activity of the essential oils of these plants against *Trypanosma brucei* TC221 and determine their selectivity with Balb/3T3 (mouse embryonic fibroblast cell line) cells as a reference. Essential oils from *A. indica*, *A. daniellii*, and *E. giganteus* proved to be the most active ones, with half maximal inhibitory concentration (IC_{50}) values of 15.21, 7.65, and 10.50 μg/mL, respectively. These essential oils were characterized by different chemical compounds, including monoterpenes and sesquiterpene hydrocarbons and oxygenated sesquiterpenes. Some of their main components were assayed as well on *T. brucei* TC221, and their effects were linked to those of essential oils. In this way, the research partially confirmed the ethnopharmacological indications, validating their traditional use and confirming the utility of popular information in the search for useful plants.

The synergic action of binary mixtures of similar constituents of essential oils against larvae of the filariasis vector *Culex quinquefasciatus* was also the inspiration behind research (Benelli et al., 2017a,b) on four Apiaceae species: *Trachyspermum ammi*, *Smyrnium olusatrum*, *Pimpinella anisum*, and *Helosciadium nodiflorum*. Initially, all the essential oils proved to be highly toxic to the larvae, but short-term exposure to both binary mixtures strongly reduced emergence rates, fertility, and natality of the *C. quinquefasciatus* that survived after the treatment at the larval stage. In addition, larvicidal acute toxicity of essential oils main constituents, i.e., germacrone, isofuranodiene, and (*E*)-anethole, were carried out, with LC_{50} being $18.6\,mg\,L^{-1}$, $33.7\,mg\,L^{-1}$, and $24.8\,\mu L\,L^{-1}$. The results demonstrated the promise of these essential oils and their constituents to develop cheap and effective mosquito larvicides.

In another paper (Pavela et al., 2016b) published in the same year, the vector target was the same but the selection of the plant totally different, as endemic to Madagascar. The reason is that in some parts of the world, there are interesting examples of endemic flora whose species could contain different essential oils and therefore different activity. For this reason, pharmaceutical companies often explore remote parts of Amazonia or isolated zones in search of new active compounds. There were examples of exploitation of rare African *Rauwolfia* species to obtain their indole alkaloids. Working with endemic species is important considering that in many cases, populations are in limited numbers and at risk of extinction, and we need to identify their molecular treasure before they disappear.

This was also the motivation for my trips to several parts of the world, focusing in particular on deserts and islands, in search of special plants. Madagascar's fauna and flora are diverse and unique. When the unique Gondwana continent braked up in several pieces, India started to move to Asia living Africa. However, a consistent block remained near to Africa, becoming a great island, now known as Madagascar. This happened more than 100 million years ago. The isolation of Madagascar gave rise to a particular case of biodiversity. This is the story of the beginning of Madagascar, as far as we know. Here, it is important to report that the island is characterized by at least seven very different habitats, each with different endemisms. The potentiality of *Cinnamosma madagascariensis*, an endemic species widely present in the forests of Madagascar, was reported to us thanks to the exceptional collaboration with Professor Philippe Rasoanaivo, who had a deep knowledge of the flora of the island and their economic importance. This plant has important traditional uses ranging from management of dementia, epilepsy, and headache to malaria (Rakotosaona et al., 2015). Few data have been reported about the chemical composition of its essential oils, and no studies have been published on its bioactivity against mosquitoes. Once again, we first investigated the chemical composition of essential oils extracted from stem bark and leaves of the plant, and later their larvicidal potential against the filariasis vector *Culex quinquefasciatus*. The reason was that when you have little information, you must consider that different parts of a plant can contain very different essential oils. In fact, GC-MS analysis revealed differences between the volatile profiles of leaves and bark oils. In the former, linalool (30.1%), limonene (12.0%), myrcene (8.9%), and α-pinene (8.4%) were the major constituents, while in the latter one, β-pinene (33.3%), α-pinene (19.3%), and limonene (12.0%) were the most representative compounds. Acute toxicity experiments conducted on larvae of the filariasis vector *C. quinquefasciatus* led to an LC_{50} of 61.6 and 80.1 $\mu L\,L^{-1}$ for the bark and leaf essential oils, respectively. Overall, *Cinnamosma madagascariensis* bark and leaf essential oils against filariasis vectors proved to be promising, since they are effective at moderate doses.

The insecticidal activity of the essential oil of another Malagasy plant was also studied (Benelli et al., 2020). *Hazomalania voyronii* is popularly known as hazomalana and its use to repel mosquitoes and resist against insect attacks has been handed down from generation to generation in Madagascar. The property of the essential oils obtained from the stem wood, fresh and dry bark of *H. voyronii* were able to repel important mosquito vectors (*Aedes aegypti* and *Culex quinquefasciatus*). Furthermore, the toxicity of the aforementioned essential oils was investigated by WHO on three insect species of agricultural and public health importance (*Cx. quinquefasciatus*, *Musca domestica*, and *Spodoptera littoralis*), respectively, as well as the adequate topical application methods and compared with the commercial repellent *N*,*N*-diethyl-*m*-toluamide (DEET). Repellence assay revealed almost complete protection (>80%) from both mosquito species for 30 min when pure fresh bark essential oil was applied on the volunteers' arms, while DEET 10% repelled more than 80% of the mosquitoes up to 120 min from application. The research validated the traditional use of the bark essential oil to repel insects, although an extended-release formulation based on *H. voyronii* essential oils is needed to increase the repellent effect over time. Furthermore, it evidenced the wide spectrum of insecticidal plants potentially useful in the fabrication of green repellents and insecticides useful to control mosquito vectors and agricultural pests, avoiding the utilization of synthetic products.

Another interesting study (Benelli et al., 2017b) was dedicated to *Helichrysum faradifani* (Asteraceae), which is a perennial endemic shrub growing in rocky and sandy places of Madagascar. The ethnopharmacological about Malagasy traditional medicine reports that this plant is used as a wound-healing agent, disinfectant, and for the treatment of syphilis, diarrhea, cough, and headache. The chemical composition of the essential oil distilled from the aerial parts of *H. faradifani*, and analyzed by GC-MS, evidenced that monoterpene hydrocarbons (51.6%) were the major fraction of the essential oil, with bicyclic α-fenchene (35.6%) being the predominant component. Sesquiterpene hydrocarbons (34.0%) were the second major group characterizing the oil, with γ-curcumene (17.7%) being the most abundant component. Its insecticidal activity was evaluated against second, third, and fourth instar larvae of the lymphatic filariasis vector *Culex quinquefasciatus* by acute toxicity assays. The most sensitive were second instar ($LC_{50} = 85.7\,\mu L\,L^{-1}$) larvae. For the third and fourth instar larvae, the estimated LC_{50} were 156.8 and $134.1\,\mu L\,L^{-1}$, respectively.

Finally, a different approach to volatile substances was performed, considering that smoke is often traditionally used against mosquitos (Ansari and Razdan, 1996). Therefore, the larvicidal, pupicidal, and smoke toxicity of *Senna occidentalis* and *Ocimum basilicum* leaf extracts against the malaria vector *Anopheles stephensi* were evaluated (Murugan et al., 2015). In larvicidal and pupicidal experiments, *S. occidentalis* LC_{50} ranged from 31.05 (I instar larvae) to 75.15 ppm (pupae), and *O. basilicum* LC_{50} ranged from 29.69 (I instar larvae) to 69 ppm (pupae). Smoke toxicity experiments conducted against adults showed that *S. occidentalis* and *O. basilicum* coils evoked mortality rates comparable to the pyrethrin-based positive control (38%, 52%, and 42%, respectively). Furthermore, the antiplasmodial activity of these plant extracts in antiplasmodial assays was evaluated against chloroquine (CQ)-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*. The *S. occidentalis* 50% inhibitory concentrations (IC_{50}) were $48.80\,\mu g\,mL^{-1}$ (CQ-s) and $54.28\,\mu g\,mL^{-1}$ (CQ-r), while those for *O. basilicum* IC_{50} were $68.14\,\mu g\,mL^{-1}$ (CQ-s) and $67.27\,\mu g\,mL^{-1}$ (CQ-r). The high potentiality of the reported data must be considered. These smokes, as the essential oils, can be quite easily obtained in good quantity and low cost and therefore locally produced and directly utilized. This is important for countries with limited economic resources.

The natural range

The distribution of individuals in accordance with the Boltzmann curve is the result of the current chemical-physical environmental pressure, concentrating the organisms of the species in the most adapted form. However, sooner or later situations are destined to change, and some of the individuals confined in the wings of the curve are ready to profit of the change and enter in the center, as soon as the conditions will be favorable to them. Another consequence of this typical statistical distribution is the careful preservation of individual types inside the population. In practice, on the genetic point of view, the best species or the favored community do not exist in absolute, and any declaration or pseudo-scientific argumentation about the primacy of a race, also human, must be considered as a guilty stretch. As confirmation, this is also in accordance with the distribution of the constituents of matter at a subatomic level. The final consideration is that the chemical composition of a plant is limited, being the expression of the genome of the species, but it may change at any time in response to internal and external stimuli.

Let us use these concepts to evaluate insecticides used in insect-borne diseases. The interest in the use of biocidal products of natural origin began in the 1930s and grew until the 1950s, when it was obscured by the arrival of synthetic insecticides on the scene. For a long time, the pesticides scenario was dominated by synthetic products, until several factors caused a decline in their utilization. However, in the last 20 years, interest in natural products has reappeared intensely, especially for the control of noxious insects at larval stage. This situation has matured, as is well known, following the indiscriminate (and not always necessary) use of excessive amounts of pesticides which, once released into the environment, are difficult to eliminate, as evidenced by the paradigmatic case of DDT (see Chapter 1). At the same time, incidence of insect resistance has increased, resulting in partial product inactivity and/or increasingly massive dosage requirements. All this led to a need for the formulation of a new generation of pesticides, and to focus research and production efforts on natural products. In 1997, the World Health Assembly reported in Resolution 50.13, Section 2.4, the need to develop bio-insecticides. Slowly but inexorably, the pesticides market registered the rise of biopesticides from natural products.

The change in favor of natural products is the result of two concomitant facts: the evidence of the environmental damage due to massive utilization of synthetic products, and a new and growing sensibility in favor of respect for habitats, asking for more compatible solutions. In the current search for the production of a new generation of pesticides, useful for mankind's battle against superbugs and other threats, to face challenges to food supply and health, plant sources play a relevant role. The current prevalence of natural products evidences that a consistent number of biocides and antibiotics have been obtained from substances produced by living organisms, which are part of the great book of Mother Nature, whose lessons are still useful. That probably means that attention in chemistry is finally moving from the free synthetic approach to natural products, already selected during the long story of molecular evolution.

In an article that appeared in ACS' *Journal of Natural Products*, Charles L. Cantrell and colleagues pointed out the impact of natural products—substances produced by living plants, animals, and other organisms—on the production of pesticides. The article reports the percentages for registered insecticides obtained from 277 new active ingredients in the period 1997–2010 (Cantrel et al., 2012). The paper's aim was focused on the impact of natural product and natural product-based pesticides on the U.S. market, obtained on the basis of NAI registrations of new active ingredient registrations with the U.S. Environmental Protection Agency (EPA). The ingredients are categorized into four categories: biological (B), natural product (NP), synthetic (S), and synthetic natural derived (SND). In particular, NPs are considered substances produced by living plants, animals, and other organisms. The report evidences that NPs, SNDs, and Bs all have origins in natural product research. NPs accounted for 35.7%, Ss for 30.7%, Bs for 27.4%, and SNDs for 6.1%, arising from the combination of conventional pesticides and biopesticides. In the registered conventional pesticides, the category of biopesticides alone registered an evident majority of NPS (with 54.8%), followed by Bs (44.6%), SNDs (0.6%), and Ss (0%). In contrast, on the conventional pesticides alone, the category S clearly dominated with 78%, followed by SND with 14.7%, NP 6.4%, and B 0.9%. The review indicates that in the same period, more natural products were registered as NAIs for conventional pesticides and biopesticides than any other type of ingredient. The authors report that when biological ingredients and natural products recreated in laboratories are included, more than 69% of all NAIs registered in that time frame have natural origins. More than two out of every three new insecticides approved in the last years are directly derived from natural substances produced in plants or animals or have significant roots in them. It is noteworthy that these numbers are very similar to those obtained if we compare with a similar projection concerning registered medical drugs in a similar period, and published in the same scientific journal.

It is also noteworthy that a similar trend can be observed in the case of the registration of medical drugs in the period 1981–2014, as previously reported in the same scientific journal by David J. Newman and Gordon M. Cragg (Newman and Cragg, 2016): 36% of registered drugs directly or indirectly derived from NP of secondary metabolism, 16% from B, 11% from SND, and only 31% from S. Again, the details reveal differences in the sectors, with NP and B dominating in anticancer and antibiotics, whereas the opposite concerns antiinflammatories, with S clearly dominating. These data, obtained on a total number of 1562 new approved drugs, are the results of several reviews that confirmed these percentages. In particular, the authors stress the role of microbes in the production of new drugs derived from natural products: "We wish to draw the attention of readers to the rapidly evolving recognition that a significant number of natural product drugs/leads are actually produced by microbes and/or microbial interactions with the "host from whence it was isolated," and therefore "it is considered that this area of natural product research should be expanded significantly."

In other words, the future of pharmaceutical drugs could be related to natural products obtained by natural synthesis. Once conceivable that the shift from synthetic pesticides to biopesticides seems to be incontrovertible, as fueled by the resistance phenomenon and the general tendency for "natural," the key argument is the choice of the raw material for the best bioinsecticide. As evident from the above reviews, bioinsecticides could be extracted from a living organism, like a plant, or produced by a living organism, like a bacterial strain by hemisynthesis, or obtained by synthesis in accordance with the structure of the active natural product. Here, the debate is open between those affirming that "a molecule is a molecule" and those in favor of "original" natural products. Anyway, in the case of an extract, the complexity of ingredients cannot be reproduced or performed by synthesis.

The main characters of a natural "ideal" insecticide should be: biodegradability, environmental care, sustainability (obtained from renewable materials) and selectively (harmful to beneficial insects). It should also satisfy some conditions to be economic appealing and relevant, like be easy to produce, low cost, derived from raw materials that available and abundant in the country where the insecticide should be utilized. The last conditions are important to avoid accumulation by multinational agencies, as is happening for coffee and cacao. Finally, but not in order of importance, the ideal natural insecticide must be able to compete in the market with the insecticides currently in use. The research for the ideal bioinsecticide is open, starting from the plant to be used.

The neem's world

Scientific classification of neem

Reign: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Clade: Angiosperm Eudicotyledons
Superorder: Rosidae
Order: Sapindales
Family: Meliaceae
Genus: *Azadirachta*
Species: *A. indica* A. Juss, 1830
Synonymous: *Azadirachta indica* var. *minor* Valeton; *Azadirachta indica* var. *siamensis* Valeton; *Azadirachta indica* subsp. *vartakii* Kothari, Londhe & N.P. Singh; *Melia azadirachta* L.; *Melia indica* (A. Juss.) Brandis

My interest in the neem tree started several years ago from curiosity. I asked myself about the most important medicinal plant worldwide. Consulting the literature, it was evident that this role should be assigned to neem. At that time, the tree was practically unknown in the Occident, since its distribution and utilization were confined to the Indian subcontinent. Therefore, I wrote an article about neem to explain the importance of this plant, and some years later, I was contacted by an Italian industry using neem oil because of the curiosity and interest raised by that article.

The references about this plant and its use appear since time immemorial, as reported in the Ayurveda and Unani systems of traditional medicines, and even in the earliest Sanskrit writings referring to the medical uses of fruits, seeds, oil, leaves, roots, and bark (Gupta, 2001). Neem can be found all over the Indian subcontinent since the time of the Sanskrit-speaking Aryans. So important was the species for the Aryans, they even mentioned and included it in their sacred *Ayurveda*, which is the sacred book of Indian medicine. The species was later dispersed throughout the Old Tropics, including Indonesia, either naturally or brought back by the ancient Austronesian sailors after visiting and trading in India at least around 2000 years ago. Through the centuries (Kumar and Navaratnam, 2000), the medical importance of neem never waned in the Indian subcontinent and it is now considered the "Village Pharmacy" for its importance in the ordinary life of Indians, who use this plant to treat several illnesses (Nix, 2007; Girish and Bhat, 2008). Many other news and references increased my interest.

The marvelous tree, the problem-solving tree, the divine tree, India's tree of life, Nature's drugstore, the pharmacy tree, the panacea for all diseases—these are just some of the terms used to evidence the respect for this plant and its importance (Ruskin, 1992; Brahmachari, 2004; Puri, 1999; National Research Council, 1992). Neem's relevance and its beneficial properties has been reported by the WHO/UNEP (1989), which considered neem as an effective source of environmentally powerful natural pesticide and one of the most promising trees of the 21st century for its great potential in pest management, environmental protection, and medicine (Nicoletti and Murugan, 2013; Koul and Wahab, 2007). Furthermore, the U.S. National Academy of Sciences dedicated a report to neem, significantly titled "Neem—A Tree for Solving Global Problems" (NAS, 1992). The importance of neem has increased exponentially in recent years. Considering the enormous quantity of results and scientific data concerning the validation of medicinal and biological properties, the international scientific community included neem on the list of the top 10 plants to investigate and use for the sustainable development of the planet and the health of mankind (Tewari, 1992; Foster and Moser, 2000). However, in the Occident, insecticidal activity is the most common application for neem oil and its derived products.

The plant, besides neem, is also known as nimba, nimtree, margosa, and Indian lilac. In botany, neem is *Azadirachta indica* A. Juss and belongs to the Meliaceae family. Meliaceae are Angiosperm Rosidae, closely related to Simaroubaceae and Rutaceae (Schumutterer, 2002). The family includes 51 genera and c.600 species. These are woody plants, like trees, shrubs, and shrublets, pantropically present, with a few temperate representatives in China and South Africa. It is estimated that c.39 million years ago, two subfamilies diverged, Cedreloideae with 14 genera and Melioideae with 37 genera, including *Aglaia*, dominating with c.120 species, and *Azadirachta* with only two species. Meliaceae are commonly known as the mahogany family, being known mainly for some important timber species, such as the true mahogany (*Swietenia mahagony*). However, losses due to overexploitation and genetic erosion, as well as toxic effects on workers, limited the use of this true mahogany, nowadays widely replaced by Spanish mahogany (*S. macrophylla*).

Azadirachta indica is an evergreen tree that grows up to a height of 15–20 m, but in favorable conditions it can to a height of about 20–35 m, with a trunk diameter up to 2.5 m (Fig. 7.3). The leaves of neem, composed of 9–19 leaflets, meaning 4–8 leaflets with a single terminal, are abundant, suspended by a strong and long petiole which lacks stipules, crowded near the ends of the branches. The leaflets are toothed, deeply serrated, their margins irregularly serrated, sharply pointed, and curved like a scythe. Young leaves are pale, tender green, and tinted with rust, but during the favorable season the tree profits from the fresh, green color and shining surface of the leaves, giving a delicate and charming appearance. White small flowers are abundant, very fragrant, bisexual, or staminate in male exemplars.

Fig. 7.3 The neem tree.

They are arranged in clusters at the axils of the leaves. They present five separated petals arranged in the form of a star. They appear in spring, and open in the afternoon giving out a delicate smell, which increases during the night. The fruit is a smooth, yellow-green, small, round drupe with a sweet-flavored pulp. During the monsoon, when the flowers have fallen and the tree is in full foliage, the curved, toothed leaves, massed round the branches, have a distinctive appearance, which is easy to recognize. From March to May, the flowers, with five whitish petals, appear in great numbers on long, drooping stems. Flowers are used to produce a bitter honey. The fleshy fruits are purplish-black, single-seeded drupes, which turn yellowish when ripe. Elliptical in shape, they have a sweet-tasting juice loved by birds and bees. However, after the rains, the fruits change, giving off a strong unpleasant smell. In autumn, fruits fall down in great quantities if not harvested.

The tree is believed to be native at least of North-East India and Burma, or Indonesia, but now widely distributed in the Indian subcontinent, and it grows naturally throughout the dry regions of the country. It is usually planted along roads and avenues in towns and villages, because it grows fast and easily, and has an irregularly rounded crown with a canopy of leaves, making it a useful shade tree (Fig. 7.4). The central regions of India are considered the patria of neem. If you go to Coimbatore, in Tamil Nadu, you can find neem trees everywhere, in towns and in the countryside. It is mainly used for shade, lining streets or in most people's back yards. In India, it grows throughout the states of Uttar Pradesh, Bihar, West Bengal, Orissa, Delhi, Maharashtra, Gujarat, and Andhra Pradesh, but the original natural distribution is obscured by widespread cultivation. Cultivation is easy, since neem usually grown from seed but can be propagated also from cuttings or root suckers, and it is a fast-growing species. Potential utilizations of neem concern human, animal, and environmental health. The last one is a recent but very important acquisition. Neem is cultivated for two main reasons: environmental care and the production of seed neem oil.

Environmentally beneficial neem

The tree's tolerance and adaptation to hot and dry climates has made it one of the most commonly planted species in arid and semi-arid areas (Tiwari et al., 2014). The survival capacity of neem is mainly due to its highly expanded root system. Neem trees are extremely useful to counteract desertification and furnish the only source of wood in arid and nutrient-

Fig. 7.4 Neem trees are often used to arborize streets and squares in towns.

deficient zones. The plant does not need particular care and grows rapidly up to 30 m tall. Neem trees attain maturity in 6–7 years in areas where the sunlight is intense, weather is warm, and good well-drained soil is found. The tree is stable in windy zones and can live for 200 years or more. To survive in arid climates, neem depends on a wide strong root system with a deep tap root and extensive lateral roots, which are ideal for soil conservation.

Furthermore, its planetary presence can contribute to positive carbon sequestration to minimize climate changes, considering that adult neem trees can retain ± 2.2 g of CO_2 per m^2 and per hour, which means 40–50 tons of CO_2 per hectare. Therefore, neem trees can be considered to be air purifiers as well as air fresheners. For all these reasons, neem is widely cultivated in warm countries, and its areal distribution is expanding rapidly by massive cultivations in sub-tropical regions of America (Caribbean Cuba, Central and Southern America), Asia (Nepal, Pakistan, Bangladesh, Sri Lanka, Myanmar, Thailand, Malaysia, Indonesia, Iran, China, Turkey, Indonesia),

Table 7.1 Current data on neem presence in many countries.

Country	**Number of neem trees (million)**
India	75–80
China	20–25
Australia	1.2
Myanmar	1.1
Dominican Republic	0.75
Thailand	0.75
Indonesia	0.6
Haiti	0.6
Brazil	0.5–0.7
Rest of the world	3.5–4.3
Total	*104–115*

Africa (Kenya, Cameroon), and Australia. The cultivation of neem trees is in particular increasing in the drought-prone areas, like in South Arabia (Arafat Valley) and in the UAE. In 1978, northern Nigeria, thanks to the governmental project Arid Zone Afforestation (AZAP), saw 700,000 neem trees being planted. In Europe, some cultivations are reported only in Southern Spain and Portugal (Sara and Folorunso, 2002).

The presumed current global neem trees presence and production are reported in Table 7.1. The data were obtained by cross-referencing several sources and are only indicative, considering that a real census was never completed in many countries (in particular in China) and many plantations are in progress. India is still by far the homeland of neem, but the scenario has changed rapidly in the last years and will continue to do so in the future, as can be deduced from the data in Table 7.1.

Visiting Oman, I noted the presence of planted neem trees in areas where acacia was the only other woody plant (usually the only plant) able to survive. The neem tree is resistant to drought and it grows in many different types of soil, but it thrives best in well-drained deep and sandy soils. Normally, it flourishes in areas wherein the neem is a life-giving tree, especially for dry coastal, southern districts. Its capacity to survive in arid zones improved cultivation in sub-arid to sub-humid conditions, with annual rainfalls between 400 and 1200 mm. It can tolerate high to very high temperatures, but it does not tolerate temperatures below 4°C, making cultivation in temperate climate very difficult. However, the future worldwide distribution of neem is not predictable. Indians are convinced that it will be difficult to obtain similar ideal conditions to their country and others in the Orient; however, the story of the cultivation of Cinchona by Dutch botanists in Indonesia tells us that the results of the cultivation can be successful and the results even superior.

In the complete Linnaean binomial name of neem we found A. Juss, which is the mark of the author of this species, designating the scientist who first published the name and a complete and scientifically reliable description of the species. A. Juss refers to Antoine Laurent de Jussieu (1748–1836), a great French botanist, who was the first to publish a complete and valid natural classification of flowering plants, named *Genera plantarum*, published in 1789, the same year as the French Revolution, surpassing the sexual system presented by Linneus. Antoine Laurent Jussieu was the member of a family of a plant enthusiasts; his uncle was the botanist Bernard de Jussieu, whose transferred knowledge and unpublished work were the starting point of the book of Antoine Laurent, and his son Andrien-Henri also became a botanist. The merit of his work was the use of multiple characters to define taxa. In this approach, he achieved a significant improvement over the "artificial" system of Linneus, mainly based on the number of the reproductive characters, i.e., stamens and pistils. Many people know the work and name of Linnaeus, but the impact of Jussieu's work was fundamental in taxonomy, founding the principles that served as the foundation of plant classification in a natural system. Many present-day plant families are still attributed to him, as the species is to Linnaeus.

That's what who can find in ordinary sources of information. The consequent idea is that Jussieu, though living in France at a historical revolutionary time, was totally dedicated to botany, but his work was also revolutionary, through a strange pathway. Deeper information about his life is a source of important lessons. His uncle, Bernard de Jussieu, invited the young Antoine to Paris, where he was trained in medicine for 4 years. However, his uncle, via his position as a demonstrator at the Jardin du Roi (Royal Garden), had other plans for Antoine. He guided his nephew's studies and prepared him for a lecturer's position at the Garden, which was soon to become vacant. At just 22-years old, Antoine was transferred to that position and his botanical training was limited because the subject then was viewed only as an accessory to his medical course. The inexperienced Jussieu had to study botanical topics by night, since he had to teach by day. Using the plants in the garden to teach plant morphology, which were arranged according to the current artificial system of Joseph Pitton de Tournefort, Jussieu started to realize the inadequacy of that system. This progressively changed his interest into a true passion for botany, and classification began until, as part of an application for a place at the Academy of

Sciences, he produced a treatment of the Ranunculaceae, starting a complete revision of the plant taxonomy system. Continuing in his study, it became apparent to Jussieu that the artificial system of Tournefort was inadequate, and from 1774 he began arranging the plants in the Royal Garden in his own way and finally transferring the knowledge in the construction of his own system of plant classification. Despite the initial success obtained by Linnaeus' sexual system, it was clear to Jussieu that the Swedish naturalist had used a counting method, whereas it was necessary to grade the characters, considering some of them more important than others, depending on how variable they are within a species. This was a necessary lesson to consider the variability of living organisms correctly. However, he continued practicing medicine, chiefly devoting himself to the health of very poor people. In 1790, he was put in charge of the hospitals and charities of Paris. In the final years of his life, Jussieu, by then almost blind as well as deaf, dedicated the last part of his extraordinary life to meditation and prayer.

Also neem can be in danger	
Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Hemiptera
Family	Diaspididae
Tribe	Aspidiotini
Genus	*Aonidiella*
Species	*A. orientalis*

The neem tree has few diseases and enemies (Boa, 1995; Schmutterer, 1998). In general, it is considered a very resistant and healthy plant (Nicoletti et al., 2017). However, it is possible that after its spread around the world, something is changing. We must move our focus from India to the new settlements, in Africa. In Northern Nigeria, neem now is planted in towns and villages, as a highly evaluated source of shade and firewood, as well in the establishment of shelterbelts. In Nigeria, like in other parts of Africa, the small twigs of neem are used to clean and whiten teeth, in consideration of its antibacterial properties. In particular, reports of pests and damages comes from East Africa, like gall mites (*Phyllocoptes* sp.) on older plants, but the most potentially dangerous pest is *Aonidiella orientalis*, known as oriental scale (Ofek et al., 1998; Lale, 1998; Elder et al., 1998). This insect was widespread in western Kenya but is not currently harmful. Likely to be native to Asia, it has been introduced to many regions via shipments of plants and then began its slow spread. Some ports check for this and other scales in plant shipments. In Africa, neem has been widely planted in the Sahel region. Oriental scale first appeared in the Sahel during the mid-1970s and caused widespread damage to neem trees planted there. Infestations were first detected in Nigeria in 1987 along its border with Cameroon and by the mid-1990s, widespread damage had been reported to neem trees throughout northeastern Nigeria.

The oriental scale is a flattened, circular or oblong insect, about 1.6 mm in diameter. It varies in color from yellow to light reddish brown. It frequently forms large colonies and sucks the sap of small stems and branches, which is phloematic sap, rich in sugars and other organic substances. Infestations often spread to the foliage, fruits, and even seeds. Feeding damage causes the foliage to die, giving infested trees a burnt appearance. This is followed by progressive dieback of branches and eventual tree mortality. The heaviest infestations appear to be on large trees located either in marketplaces or around human settlements. The female attaches to the surface of a plant and causes the disease by the larvae, which roam the plant, feeding on sap by inserting their stylets, sucking sap, and weakening the plant progressively. The physical damage includes discoloration and deformation of leaves. Flowers and fruits fail to develop. It is noteworthy that the effects of the pest attacks are very similar to those already reported for olive trees, including exsiccation of leaves and wood. The lesson is that, in this outbreak, we have an explosive negative mixture of alien species and man's activity on the habitat. The enormous spread of neem trees in recent years is an unusual phenomenon, whose consequences should be better monitored.

Chemistry of neem

Before going into detail about the constituents, we must consider the typologies of the forms of the marketed products containing natural products. We are referring to insecticides, but the same considerations are simply applicable to nutraceuticals, phytomedicines, cosmetics, and even food. It is possible to find the plant drug, meaning a part of the plant utilized. The plant drug is usually utilized exsiccated, or as a derived product, like an extract, resin or oil, which can be obtained as such, or be enriched in one or more constituents, which are considered responsible for the activity. In this sequence, the original starting point, which makes the plant useful, has been betrayed in favor of increased efficacy. However, following this treatment, there are products registered as food supplements containing 90% of a pure substance and usually the origin is not at all natural (although this is not declared on the label). In such cases, the product is more similar to a medicinal drug than an extract and it should be considered and used in medicinal form. The distinction between such marketed products is not evident and not reported to the unaware consumer, who may well prefer the product due to its apparently "natural" origin.

Knowledge of the chemistry is therefore fundamental and the basis of any decision about the appropriate use of a plant. However, we cannot know exactly what is inside a plant. All our methods of investigation are limited and may be misleading, although papers and books are full of information about the compounds contained in plants or their derived products. This is the consequence of plants' extreme chemical complexity. In a single leaf of hemp, more than 400 constituents have been detected, considering the secondary metabolites alone, and hemp can contain high levels of THC or cannabinoids can be practically absent. In such cases, the morphology does not give any help and only a reliable chemical analysis can indicate what kind of hemp we are handling.

Since its beginning, phytochemistry has sought knowledge of all natural products. In about one hundred of years of activity, innumerable analyses were made and an enormous quantity of data collected in a very useful data base, but recent advancement in analytical devices and novel interpretation ask for a revision of the result of this job (Kaushik et al., 2014; Forin et al., 2011). We must remember that the molecular world is not detectable by our senses and therefore we have secondhand and probably only partial information. Sometimes, this information is considered sufficient to assign the compound/activity relationship, but only until other analyses confirm the presence of other molecular candidates.

The seeds of neem contain at least 100 identified biologically active compounds (Govindachari, 1992). Among them, major constituents are nor-triterpenes, named limonoids, i.e., azadirachtin, nimbin, nimbidin, nimbolides, and many others (Ragasa et al., 1997). However, each year other new limonoids are discovered. More constituents mean more possible activities.

Preparations from the leaves or oils of the seeds are used as general antiseptics (Mossini et al., 2009). Due to neem's antibacterial properties, it is effective in fighting most epidermal dysfunction, such as acne, psoriasis, and eczema. Ancient ayurvedic practitioners believed high sugar levels in the body caused skin disease. Neem's bitter quality was said to counteract this sweetness. During the last Desert Locust Plague in Africa, it was noticed that these insect, *Schistocerca gregaria* (Forskal), ate almost any vegetal around, leaving a bare landscape when they fly away, except for neem trees, probably because of the antifeedant effect of the very bitter leaves, due to the presence of limonoids. Traditionally, Indians bathed in neem leaves steeped in hot water. Since there have been no reports of topical application of neem causing adverse side effects, this is a common procedure to cure skin ailments or allergic reactions. Neem also may provide antiviral treatment for smallpox, chicken pox, and warts, especially when applied directly to the skin. Its twigs are commonly used to clean and disinfect teeth. The brushing of teeth with neem to prevent gum diseases and for teeth whitening is very common, and not only in India. There are also various kinds of natural toothpaste on the market that contain neem extracts. It is also possible to prepare a homemade toothpaste to achieve shiny, cleaner teeth. Neem powder is made by grinding dried neem leaves, which is traditionally used by mixing one teaspoon of neem powder with one teaspoon of baking soda and enough water to make a useful paste. These preparations can help to avoid the plaque and tartar that build up in gums, which are the root causes of bad breath. In addition to cosmetic uses, neem's antimicrobial activity to maintain dental health is also worth noting (Chava et al., 2012). A preparation can be obtained by boiling neem leaves in water until they reduce to a quarter of the original volume. Finally, gargling with this concoction contributes to good breath and whiter teeth, as it kills the bacteria inside the mouth. The teas of neem leaves are utilized in Indonesia and Oman for their digestive properties (Sujarwo et al., 2016).

Neem's effectiveness is due in part to its ability to inhibit pathogens from multiplying and spreading (Benelli et al., 2016). Neem produces pain-relieving, antiinflammatory, and fever-reducing compounds that can aid in the healing of cuts, burns, sprains, earaches, and headaches, as well as fevers (Chopra et al., 1952). Several studies of neem extracts in suppressing malaria have been conducted, all supporting its use in treatment. Neem has broad applications to human and animal health, as well as organic farming (Bhowmik et al., 2010). It is reported as a powerful antiviral and antibacterial, with peculiarities that set it apart from other herbs in that class of broad antimicrobials (Sandanasamy et al., 2013). Neem oil is also commonly added to a variety of creams and salves. It is effective against a broad spectrum of skin diseases including eczema, psoriasis, dry skin, wrinkles, rashes, and dandruff. These are just a few examples of the possible utilization of neem, and the potentiality of neem is considered by everybody, including the ONU and other institutions, to be very high, but so far little has been done to develop appropriate products from it to help mankind. In particular, considering insect-borne diseases, in vivo activity of neem seed oil (NSO) against malaria *Plasmodium* has also been reported (Dahiya et al., 2016; Trapanelli et al., 2016).

Today's exploding growth in human population is seriously depleting the world's natural reserves and economic resources. Unless the runaway human population growth rate is slowed down, there will be little hope for raising everyone out of poverty in the developing world. Besides educational constraints, the nonavailability of inexpensive methods of contraception, which do not cause trauma or impose on the esthetic, cultural, and religious sensitivities of people, limit the success of birth regulation programs. However, recent findings indicate that some neem derivatives may serve as affordable and widely available contraceptives. A recent controlled study in the Indian army proved the efficacy of neem as a contraceptive. In 2020, the report of the Washington-based International Food Policy Research Institute predicted a world even more unequal than the present, with food surpluses in the industrialized world and with chronic instability and food shortages in the global south, particularly in African countries.

The state of the art of neem oil

The main product of neem seed oil (NSO) is the fixed oil obtained by expressing the seeds, still enclosed in the kernels. Therefore, the fleshy pulp is removed or dried, to obtain the inner part. NSO can be obtained by different extraction methods. Most NSO is produced in India by small-scale producers at ordinary temperatures using very simple machinery, which is utilized in other periods of the years for other oily extraction, like arachnids or soya seeds (Figs. 7.5–7.9). However, modern apparatus is also used in India and many other countries are now producing and refining NSOs. Therefore, considering also the possible different geographical origin of the raw material, combined pre- and postharvesting factors can result in great differences in constituents present in marketed NSOs, as already reported.

Therefore, despite the common definition for all the oils obtained from kernels of neem, it is necessary to consider that there is no single NSO, but many NSOs differing in shape, color, viscosity, chemical constitution, and

Fig. 7.5 A farm in India producing neem oil.

Fig. 7.6 Simple apparatus for expression of neem kernels.

activity. Medicinal and cosmetic utilizations are relevant and continuously increasing. Cold pressed neem oil is commercially known as margose oil and considered as pressed directly from seeds. There are hundreds of marketed products worldwide based on margose oil. Neem, or margose, oil has a brown color, a bitter taste, and a garlic/sulfuric smell. The oil is usually obtained by simple pressing, but extraction by organic solvents, like hexane, is also used, though in such cases traces of the residue of solvent are always present in the final product. The type of insecticide is commonly

Fig. 7.7 Kernels of neem, the raw material for neem oil.

Fig. 7.8 Production of neem oil.

registered as biocide, insect repellent, and antifeedant, intended for use on outdoor and greenhouse agricultural food and ornamental crops as a repellent and insect growth regulator. The products are considered to have no risk to human health because of their low toxicity via all routes of exposure.

Fig. 7.9 Neem cake resulting from the extraction process.

There is no reason to believe that any nontarget organisms, including honeybees and other beneficial insects, would be adversely affected, as tested by the Environmental Protection Agency (EPA).

Industrial applications of neem oil

The Environmental Protection Agency (EPA) is probably the most important agency in charge of environmental care and health. It is an agency of the United States federal government whose mission is to protect human and environmental health. The EPA is also in charge of the regulations of carbon emissions from power plants, automobiles, and other contributors to climate change. The EPA became popular in Europe because of a civil enforcement case against Volkswagen and other car manufacturers, subject to reservations set forth in each of the partial settlements. In 2017, the U.S. Department of Justice resolved a criminal case against Volkswagen AG with a plea agreement for the offenses of conspiracy, obstruction of justice, and entry of goods by false statement, and the U.S. Customs and Border Protection resolved civil fraud claims with Volkswagen arising from the illegal importation of affected vehicles.

The EPA was established in December 1970 by an executive order of President Richard Nixon, with headquarters in Washington, D.C., in response to widespread public environmental concerns that gained momentum in the 1950s and 1960s. EPA is a giant public agency with a budget of 9 billion US $, and it is born as reaction to the public movement in favor of the environment due to the Carson's book (see Chapter 1). It is considered reliable because independent. The EPA is responsible for creating standards and laws to protect and preserve the natural environment and improve the health of humans by researching the effects of and mandating limits on the use of pollutants. The EPA's aims include the regulation of the manufacturing, processing, distribution, and use of chemicals and other pollutants. In addition, the EPA is charged with determining safe tolerance levels for chemicals and other pollutants in food, animal feed, and water.

The best presentation of neem insecticide properties and the rationale of its utilization is the report of the EPA about the registration of cold pressed neem oil, concerning a product named plasma neem. The report has a significant subtitle: "Reasons Why Neem Oil is an Effective Way to Control Insect Hoppers" (EPA (US Environmental Protection Agency), 2012).

The target of the product cold pressed neem oil is insect hoppers, because of their special liking for cash crops. The consequence is a crisis because of insect hoppers infesting these cash crops by chewing and sucking the leaves, as reported by many farmers. The reasons to use neem are focused on the effects of chemical fertilizers, which "provide a remedy but tend to kill beneficial insects as well." The report identifies three reasons why neem oil should be considered an effective way to control insect hoppers which feed on cash crops.

Reason 1: Selectivity. "Neem oil is usually sprayed on the leaves and stalks of a cash crop. So it is aimed at only the insect hoppers who spoil the cash crop by chewing the leaves or biting off bits of the stalk. The insect hopper which attacks the plant by consuming it will end up consuming the neem oil as well and hence die as an aftermath. Beneficial insects, who replenish the soil in a natural fashion, do not consume the plant and hence do not get affected by neem oil."

Reason 2: Eco-friendly efficacy. The content of azadirachtin in neem oil is considered very good at controlling and eliminating insect hoppers, avoiding the negative effects on the productivity of cash crops. Furthermore, the neem oil exerts no damage to the soil or disruption of the chemical composition of a fertile soil. The product affects biological functioning of the insect hopper, and therefore the insect hopper forgets to feed or breed after devouring a cash crop with neem oil sprayed on it. The conclusion about this point is clear: "This leads to the complete elimination of the insect hopper and the infestation cycle without any other adverse chemical side effects." The action is mainly larvicide, but also deterrent and adult insecticide in most species.

Reason 3: Cost-effectiveness. The utilization of neem oil for preserving cash crops from insect hoppers is considered very cost-effective. In comparison to chemical fertilizers, neem oil is expensive, but other positive effects must be evaluated. There is another consideration about the cost/benefit effect: eliminating negative insects and worms by neem oil, farmers need not spend money on buying supplements for the enrichment of soil. The heavy investment to preserve soil quality is totally avoided when a farmer uses neem oil. In conclusion: "Overall, it can be said that neem oil as a source to control insect hoppers in cash crops is extremely beneficial for farmers."

The final consideration of the EPA is the advice not to use the content of the report as an endorsement of NSO. In fact, it is important to remember that the report is based on scientific and experimental data. The EPA is not working in favor of the industry or the market, but its judgments must be considered objective although its aim is environmental improvement. NSO, obtained by the cold expression method, is the only natural product-derived insecticide, whose registration was approved by EPA. The EPA's authority is so far internationally recognized.

Therefore, in the report there are three main key items of information: the evaluation of the selective insecticide activity; the preference for synthetic products in consideration of the eco-friendly properties; and the indication of azadirachtin as necessary for the activity. Let us consider the report as a guide and evaluate each of these points. Later, we will add other considerations.

It is noteworthy that all these indications can be found already reported and present in the information about the ethnobotany and traditional medicine of neem. In the cultivation of rice, when the plantlets are underwater, the addition of extracts of neem to the usual fertilizer, not only decreases the mosquitoes number, but also has beneficial effects on the production. The utilization of neem to increase soil fertility and treat medicinally plants and livestock is recommended in the *Upavanavinos*, an ancient Sanskrit book on agriculture. The dried leaves and the oil are used as preservatives against insects and microorganisms for the postharvesting conservation of foods. Conservation is guaranteed for more than a year. Panels derived by the extraction of the oil are used as food additives for livestock, and the animals are washed with this diluted oil to prevent the attacks of parasites or harmful insects.

In the last decade, the focus was on the potentiality of a new analytical technique, HPTLC (high performance thin layer chromatography)

Fig. 7.10 Part of the HPTLC devices.

(Fig. 7.10). HPTLC is the last evolution of planar chromatography and allows the evidence of most of the constituents of an extract in an identifying track, named fingerprint, wherein identification of constituents can be obtained by direct comparison in the same plate with the correct standards, utilizing the Rf value and the reaction with adequate derivatization (Nicoletti et al., 2012a,b). Improvements in separation and visualization of the spots are obtained by reduction of the size of the particles of the silica gel, constituting the fixed phase. The mobile phase can be selected on the ample repertory of solvents and their mixtures, as well as the several methods of derivatization and detection. The final effect, in comparison with ordinary planar chromatography (TLC), is like a myopic person wearing glasses. The advantages in comparison to the TLC are in the total control of the environmental conditions and the automatization of the procedures. Each step of the analysis is performed entirely by a series of devices, and the operator is only asked to produce the program of actions by the software. Plates can be visualized and derived in several ways, obtaining multiple information (Figs. 7.11 and 7.12). They can be easily preserved and stored as digitalized images inside the computer, and immediately sent everywhere or compared with a data bank. However, the most important feature of HPTLC is that the results of the analyses are very clear, thanks to careful preparation work that enabled optimal chromatographic conditions, as demonstrated by the quality of the images. In other words, our idea was to "see the molecules" (Nicoletti, 2013) and obtain simple and clear evidence of the

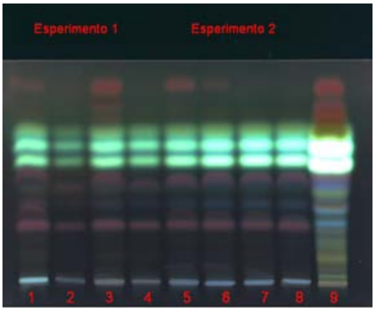

Fig. 7.11 A HPTLC analysis of NSOs, demonstrating the different constitution of marketed oil neems.

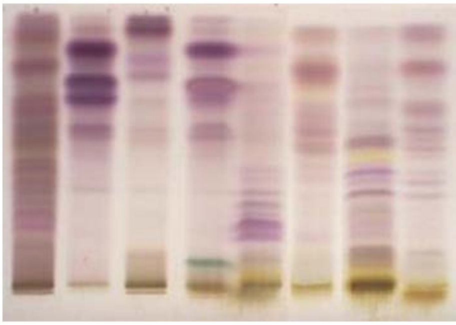

Fig. 7.12 Another HPTLC plate shown in a different way.

metabolic production. Molecules are too small to feel their presence with our senses or directly by any sort of device, but it is possible to evidence their chemical properties in the plate, recording the Rf value, the fluorescence, and the color after derivatization. An HPTLC or NMR graphic needs an expert for correct interpretation, such as any specialized analysis, like a cardiogram (Nicoletti and Toniolo, 2015). In HPTLC, without any knowledge of chemistry, the presence or absence of a determined spot is evident (Figs. 7.11 and 7.12).

HPTLC was selected to obtain a metabolomic approach, meaning the study of many constituents as possible, focusing on secondary metabolites (Toniolo et al., 2013). Metabolomic is one of the -omic sciences generated by the dissection of the dogma of biology, based on the sequence DNA➔RNA➔proteins.

It is necessary to propose some comments about the dogma of biology and why its crisis generated a series of other points of view. First of all, the use of the word "dogma" should be avoided in biology, since the matter is more complicated than a simple sequence, as actually happened. The central dogma of biology was first proposed in 1958 by Francis Crick, as a consequence of his discovery of the structure of DNA. The dogma describes process by which the instructions contained in DNA are converted into a functional product. Another definition is: "the coded genetic information hard-wired into DNA is transcribed into individual transportable cassettes, composed of messenger RNA (mRNA); each mRNA cassette contains the program for synthesis of a particular protein (or small number of proteins)" (sources: definition from Chapter 1: The Dynamic Cell, of *Molecular Cell Biology*). The flow of genetic information within a cell follows the sequence: DNA codes for RNA via the process of transcription (occurring within the nucleus), RNA codes for protein via the process of translation (occurring at the ribosomes), and proteins are responsible for the synthesis of the other metabolites (proteins are spread everywhere). Cell data are organized within the database of DNA and reversed in the metabolic flux, through RNA. Although clearly deficient, the central dogma of biology dominated genetics for decades, but through ongoing research, many exceptions were discovered. For example, most DNA is silent, since it does not encode proteins. Retroviruses, which are relevant for our arguments, present the possibility that RNA transcribes into DNA through the use of a special enzyme called reverse transcriptase, and other cases of deviance can be reported. However, the biggest revolution consists in the direction of the arrows. It is necessary that information could follow also the reverse pathway, allowing an appropriate response by the genome potentiality. Therefore, at least the dogma must be rewritten with two-way arrows.

In principle, a metabolomics study should be the determination of the pathway of cell production from the genome through transcription, but the term "metabolome" is now used to evidence the whole pool of metabolites, in particular for natural products, whereas transcriptomics is related to proteins. Transcriptomics involves serious difficulty to obtain reliable results. A protein seems perfectly comfortable inside the cytoplasm, but outside, irreversible denaturation causes definitive degradation and consequent difficulty in understanding the protein's functionality. In contrast, small molecules are more stable in any environment and their molecular structures at least can be determined by phytochemical analysis (Nicoletti and Toniolo, 2012; Toniolo et al., 2014). However, in the metabolome we have hundreds of thousands of different constituents to be studied, and the classic approach to study the molecules one by one is impracticable, and other methods must be utilized. The lesson is that the role of any metabolite cannot be discarded a priori, and also a secondary influence in the evaluation of the property of an extract can be important to definite and obtain the final reaction. Once again, the "magic bullet" paradigm is under discussion, but the total utilization of plant extracts must also be considered an unsatisfactory solution.

The aim of our approach was to adapt the method to other subjects outside the pharmaceutical applications. Therefore, our first studies focused on the determination of adulterants in nutraceuticals and other pharmaceutical products, like the "green viagras." Later, we adapted the method and the devices to use the metabolome as a source of information about what is going on in a complex system in which living organisms are acting. Therefore, we are able to study the effect of environmental factors, like ozone, on the quality of wine (Valletta et al., 2015). However, probably the most impressive application was the study of the environmental effects of the *Costa Concordia* disaster (Toniolo et al., 2018).

On the night of January 13, 2012, the *Costa Concordia*, a giant yacht with approximately 1500 cabins, 3229 passengers, and 1023 crew, was wrecked off the rocks of the Italian coast a few hundred meters from the port of Giglio, a little island on the Tyrrhenian coast in Tuscany. Like an injured helpless mastodon, the cruise ship inclined dangerously, until the inclination stopped with most of its starboard side under water. Because of the inclination and the amount of people, the overnight evacuation of the *Costa Concordia* was a challenging process, and 32 people died. The cost of removing the ship was US$799 million. For scientists like us, interested in environmental damage, it was a unique occasion. For 1 year, 9 months, and 4 days, the enormous hull of the boat altered the underlying marine habitat, interrupting the normal flow of sunlight over a surface of more than 10,000 m^2. The seagrass *Posidonia oceanica* was chosen as the target organism of the impact evaluation, since, like in other parts of the Mediterranean Sea, it forms large underwater meadows. Using HPTLC analysis, it was possible to determine the health of each collected plant and make a map of the metabolic damage, which accorded with the shadowed area. However, albeit the negative conditions, the rhizomes turned out to be mostly still alive and able to reproduce the meadow again. Therefore, the final task of our research was simply to wait until nature carried out its work. However, there is a further chapter of this story, written after our study. To remove the ship, a platform was transported from north Europe. The problem was that the bottom of the platform was full of mytilus. When the platform was exposed to the hot Mediterranean sea temperature, the mussels died, releasing their bodies, covering down the sea background and causing a further source of damage. A clear example of human stupidity and superficiality. Anyway, devoted to our task, we are now repeating our analyses to understand what happened and what is still going on, relying on the quality and reliability of our indisputable results.

The study of neem oil was based on the experiences obtained by improving the HPTLC devices via the metabolomics approach. The central idea was to collect as much information as possible about the constituents of the neem products, without any preference for any kind of metabolite, considering any product and any extract like a unique molecular system.

In the HPTLC analyses on NSOs, the objective was to achieve the total chemical characterization of the used oil, and then the derived products, by means of the production of a chromatographic reference profile of the metabolites' production. This objective is not easy to achieve due to the complexity and variability of neem oil. Neem products are subjected to great variation in composition, due to preharvesting factors, like environmental situation, genomic differences, influences of the habitat, and others, including postharvesting situations, like harvesting and stocking, treatment of the raw material, separation of different parts, extraction methods, production of the final product, and others. In fact, analyzing different marketed neem oil from several productions and countries, we decided that it was not possible to refer to a single neem oil, but to neem oils in the plural, due the great differences in composition. Therefore, we decided to obtain and adopt a reliable reference metabolomic HPTLC profile for the neem extracts or products to be utilized in our biological experiments in vitro and in the field.

In fact, one of the typical problems in activity tests is the differences in raw material giving rise necessarily to different results in activity and utilization. Another important aspect of our metabolomic study was that the complexity of the neem profile was even greater than expected. This result is the consequence of the generalist approach. In other molecular chromatographic or spectroscopic analyses, like HPLC, the result is *sub judice* on the detector's settlement. Therefore, if the molecule does not possess the adapt chromophore, the molecule, even if it is the main component, is invisible to the detector. In HPTLC, there are universal derivatization methods, like H_2SO_4, to reveal the organic substances, but it is possible when necessary to adopt a particular agent. In this way, it was possible to exclude the presence of a relevant percentage of azadirachtins and the occurrence of other constituents relevant for the activity. This is another recurring lesson for those studying natural products. Although a plant has been the object of several phytochemical studies, new constituents can be obtained. An example is the discovery of gossypol in cotton oil.

Insecticidal activity of neem

Insecticidal activity is reported in a hundred or so published papers, concerning a wide range of species of arthropods, as confirmations of many traditional uses (Schumutterer, 1995; Amirthalingam, 1998; Jones et al., 1989; Van der Nat et al., 1991; Biswas et al., 2002). Leaves are used in houses to repel and keep away insects. When half of a sample of soya leaves are sprayed with NSO and offered as food to the Japanese coleopteran (*Popillia japonica*), the insects feed only on the nontreated parts of the leaves. In Nicaragua, farmers spray their cultures with an aqueous extract obtained by leaving the seeds for 12 h in water.

In general, NSO-based products have proven to be very effective against a huge range of pests of medical and veterinary importance, mainly including mosquitoes. The insecticidal properties of neem and its many formulations are based on experimented antifeedant, fecundity suppression, ovicidal and larvicidal activities, including growth regulation and repellence against a great number (around 600) of different insects, also at very low dosages, whereas useful insects were shown to be unaffected (Nicoletti et al., 2014; Isman, 1997; Sharma et al., 1993; Schumetter and Singh, 1995; Forin et al., 2011). The deterrent activity was also important, which can be easily determined as reported in Fig. 7.13. Other studies, like the molting and the growing of the selection under investigation, need special devices, as those reported in Fig. 7.14.

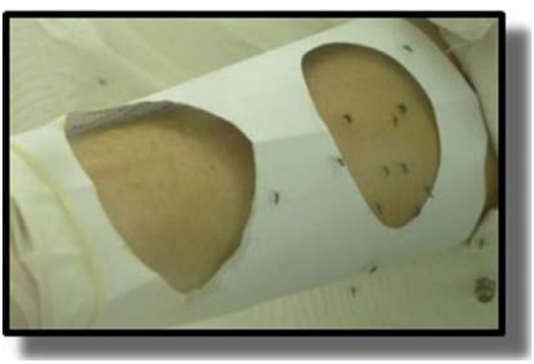

Fig. 7.13 The deterrent activity of oil neem can be easily and directly determined.

Fig. 7.14 Other activities of neem oil need special chambers to follow the growing and molting of the insects.

In particular, a concentrated extract of neem seeds, named MiteStop, developed by the university spin-off company Alpha-Biocare (Düsseldorf, Germany), proved to be very effective against a huge range of pests of medical and veterinary importance, including *Ixodes* and *Rhipicephalus* ticks, house dust mites, cockroaches (*Blatta*, *Blattella*, and *Gomphadorhina*), raptor bugs (*Triatoma*), cat fleas, bed bugs, biting and bloodsucking lice, poultry mites, and beetle larvae parasitizing the plumage of poultry. Neem leaves can also be used to protect stored woolen and silk clothes from insects.

Concerning mosquitoes, emulsified formulations of *A. indica* oil showed excellent larvicidal potential against different mosquito genera, including *Aedes*, *Anopheles*, and *Culex*, also under field conditions. Insect growth regulatory activity of neem-borne molecules alter or block the metamorphoses of larvae (Toniolo et al., 2014). Neem weakens the cuticle defense system of the young instars, causing easy penetration of pathogenic organisms, or interferes with the molting mechanism. Concerning biological control, an increase of the control of *Aedes* populations was observed after the combined application of predatory copepods and neem-based larvicidal products, since repeated application of NSO does not affect populations of predatory copepods. However, relevant limitations are related to the relatively high cost of refined products and the low persistence on treated surfaces exposed to sunlight. In the soil, the half-life of azadiracthins, meaning the time necessary to degradate the compounds, is from 48 min to 4 days, depending on the environmental conditions, like moisture, high temperature, and sun. The breakdown is faster on plant leaves, due to the exposure and the surface.

In the attempt to assign the active constituents of NSO, we must consider that the chemistry of neem is very complicated in terms of numbers and types of constituents. Despite the great quantity of dedicated research, chemical research is far from complete. Hundreds of compounds have been isolated and identified from various parts of neem, with seeds being the most investigated for their commercial value. The seeds may contain approximately 45% of a brownish yellow oil, mainly constituted by several fatty acids, i.e., oleic acid *cis*-9-ottadecenoic (50-60%), palmitic esadecanoic acid (13%–15%), stearic acid ottadecanoic (14%–19%), linoleic acid *cis, cis*-9,12-ottadecadienoic (8%–16%), and arachidic acid (1%–3%), although several other compositions have been reported. After a certain time, fatty constituents tend to separate and appear as white amorphous material. The main characteristics of the oil are its unpleasant strong alliaceous odor and acrid taste, attributed to sulfurous constituents. The shape and consistence can be very different according to the extraction method and the source. In fact, the composition of neem oil is highly variable, depending on preharvesting factors, like the cultivar, the geographic and environmental origin of the raw material, collection seasons, and postharvesting, like the extraction method, preservation, and conservation. Extraction can be executed with different apparatus, temperatures, pressures, and methods, affecting the yield as well as the content. As later reported, these aspects have been deeply considered and HPTLC analyses can be utilized to ensure the chemical composition of the neem oil utilized in the biological experiments.

Among the c.300 compounds characterized from the neem seeds, more than one-third of them are nortriterpenoids, which are triterpenoids lacking some carbon atoms (Kaushik et al., 2014). Nortriterpenoids are chemotaxomically well located in a few related families of Rosidae Angiosperm Dicotyledons, i.e., Rutaceae, Simarubaceae, Cucurbitaceae, and Meliaceae, within the Rutales order. Generally, in the plants of the Rutales order, the partial loss of the lateral chain is followed by a complicated rearranging of the remaining part, giving rise to different polycyclic molecular skeletons, full of oxygenated functional groups, partially acylated. Syntheses of complex natural compounds are costly and therefore they are usually used only for special activities. It is necessary to consider this point, which is in favor of the use of the plants as source of these compounds, since the synthesis can reproduce the chirality of nortriterpenoids only with extreme difficulty and cost.

Nortriterpenes are a very interesting part of the plant's chemical ability, that we call biosynthesis, to produce active complex molecules.

Nortriterpenes present very complicated structures and high numbers of active parts. We must remember that in natural products, activity is based on the presence of functional groups, made by heteroatoms, which means mainly O and/or N. If nitrogen is present, you have alkaloids, otherwise the range is higher, comprising phenols, alcohols, ketones, and others. However, the introduction of an oxygen inside a derivative usually is obtained to increase the activity, but also introduces instability in the molecule. We must remember that a natural product started from CO_2 and is likely to become CO_2 again at the end of its life. This is the necessary turnover of atoms and energy in organic matter. The first process accumulates energy and it is based on reductive reactions (endothermic reactions), whereas the second one is based on oxidation and produces energy (exothermic reactions). In other words, life is based on subtraction of negative entropy from the habitat, and at the end of its life, the organism releases this energy to the system. During its life, the molecule is expected to carry out its role inside the biosystem, which is the reason for its synthesis inside the plant. To understand the role, the nature of the target is essential. In insect-borne diseases, the natural product should interfere in the life of herbivorous insects or dangerous pathogens. In the case of neem, the activity is mainly larvicidal, blocking the metamorphosis to the next pupal stage. The larvae are unable to develop and change their state. To obtain this result, a lot of chemical and finalized activity are necessary, in this case consisting of hormonal interference in the insect metamorphosis process. In other words, the molecule must be able to mimic the internal complex chemical apparatus that allows drastic changes in the forms of the insect, until it stops the process.

Let us consider in particular the class of nortriterpenes. We have already had occasion to meet monoterpenes in the essential oil constituents. Owing to their biosynthetic origin derived from progressive accumulation of isoprene units, each made by five carbon atoms, terpenes are classified according to the increasing molecular weight in monoterpenes (C10), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). Squalene, the unique precursor of all triterpenes, is a linear unsaturated hydrocarbon, but its derivatives, for stability reason, are all cyclized with hexagonal and pentagonal cycles in the final structure. Among triterpenes, the most famous class is certainly the steroidal one. Steroids are present in any organisms, where they carry out several fundamental roles. Without steroids, starting from the cholesterol stabilization of cell membranes to the influence on metabolism, no cell, and therefore no organism, could survive. However, each organism synthetizes its own steroids. In fact, animals possess simpler steroids usually with few oxygenated functional groups, bacteria produces steroidal triterpenes mainly dedicated to the stability of the cell envelope, and plants biosynthetize quite complex structures, named phytosterols. Generally, phytosterols possess more cycles respect to the ordinary structure of the steroid model and an increase of the number of functional groups (Roy and Saraf, 2006). Animal steroids are based on a simple, easy-to-remember sequence of four fused cycles: three hexagonal and the last pentagonal. The basic structure of a steroid is quite easy to write and remember by students, including the stereochemistry, whereas the structures of limonoids are very complex and not so easy to remember. The problem is that in the basic structure of a steroid the sequence of the cycles is linear, whereas in limonoids there are complicated re-arrangements, causing a circular total structure. The insect's molt and metamorphosis are triggered and directed by hormones, usually consisting of steroids, such as prohormones (pheromones or juvenile hormones) and ecdysones. The term "ecdysone" was introduced by the German biochemist Peter Karlson (1918–2001) in 1956 in "Chemische Untersuchungen über die Metamorphosehormone der Insekten" (Karlson, 1956). The etymology of this word is interesting, from the Greek *ekdusis* "shedding," or more precisely ἐκ(ς) ("external" or "from inner to out") + δύω ("dress oneself") + *-si(s)* ("action") + *-ona* ("hormone"). However, ecdysones have typical steroidal structures, whereas limonoids are the result of a complex chemical rearrangement.

Thus, to interfere in insect life, special triterpenes are necessary. Several plants, like those in Rutales and Sapindales and related families, are specialized in the synthesis of such molecules, probably made to defend the plant from phytophagous insects. To obtain this result, great chemical ability is necessary. First, part of the typical hydrocarburic lateral chain, typical of most steroids, is lost ("nor" in chemistry means exactly this passage obtained by cutting C–C bonds), and the remaining part is both oxygenated and compressed in a complicated polycyclic structure, which is quite stable in the cell environment, but easily degradable in contact with atmospheric oxygen and sunlight. In this way, the nortriterpenes can be produced in the plant and transferred to the insect with mortal effects through a subtle toxic effect. The idea is to interfere with the growth regulators, interrupting the balance of hormones, named juvenile, in particular interrupting the transition process from the larval instar stages to pupae and adults by juvenile hormone analogues. Therefore, on learning this lesson from nature, we can find solutions and inspirations.

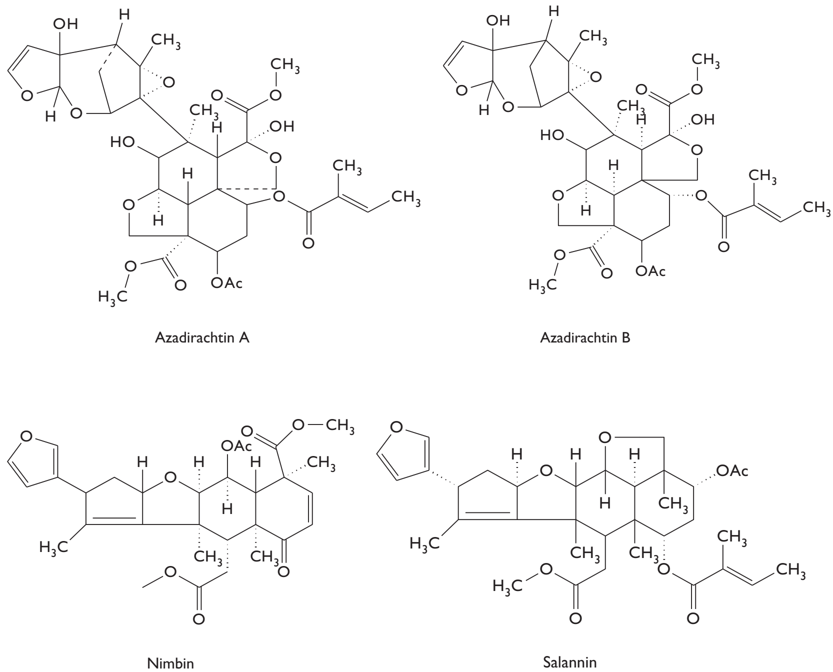

Fig. 7.15 Structures of major limonoids of NSO.

On the basis of these considerations and the structural diversity (Fig. 7.15), nortriterpenes can be divided into two main groups: limonoids (C26), with partial loss of the lateral chain (Manners, 2007), and quassinoids (C20 and C19), with total loss of the lateral chain (Vieira and Braz-Filho, 2006). In ancient times, plants containing these kinds of compounds were mainly famous for the bitterness of their drugs, utilized in the production of tonics, digestifs, and medicines. Limonoids are part of our ordinary experience with some fruits and are crucial for the dissemination process. When we eat a citrus fruit, such as a lemon or an orange, we taste the agreeable flavor of the juice, but the seeds are discarded because they contain the nortriterpene limonin, which is very bitter and unpleasant. By throwing away the seed, we contribute to the reproduction of the plant. Several other properties of limonoids have also been reported, including antioxidant, antimicrobial, and antitumoral activities, the insecticidal of neem being so far the most important.

Limonoids are considered the most active ingredient of insecticide neem products. They are classified into nine basic structures, with three main skeleton types: (a) azadiracthins, highly polioxygenated and acylated, with a saturated first ring, a tetrahydrofurane ring between the two first rings, and a final dihydrofurane ring chained with the other part of the molecule; (b) nimbins, less oxygenated and acylated with a skeleton evidently similar to that of the steroids, the furane ring with only a link with the remaining part of the molecule; and (c) a third type similar to the azadirachtins one, but the polycyclic part containing the dihydrofurane ring is less complicated, giving rise to a more linear general skeleton. Such variability is necessary to sustain the large range of targets. In fact, these differences are just as important for the biological activity as for the decomposition. However, in terms of market considerations, azadirachtins, in particular azadirachtin A, are considered the reference constituents to evaluate the quality, and therefore the activity, of neem oil.

Azadirachtin A is a highly oxygenated C-secolimonoid, whose content in the seeds is highly variable (0.1%–1%), mainly depending on the producing zone and the seasonal trend. This compound acts as a biocidal on insects after ingestion or contact, with several effects: (a) interference on the growing processes, inhibiting the molting or blocking the hormone ecdysone synthesis; (b) antifeedant, with reduction of feeding; (c) negative effects on adult fecundity and egg fertility; and (d) diminution of the defense capacity of the cuticle, easing the penetration of pathogens. In particular, the larvicidal effect consists in the formation of the "permanent larvae," i.e., larvae are able to complete the molt as a consequence of destruction of the cuticle or of hormonal perturbation of the metamorphoses. This study consists in the careful observation of the larvae transformations and in the daily count of the consequence of azadirachtins and related compounds on the molting of phytophagous with buccal apparatus, either biting-sucking and chewing, comprised in all systematic categories: orthoptera including grasshoppers, locusts and crickets, etheroptera, homoptera, aphides, cicadellidae, hymenopterous, thysanoptera, aleurodidae, dipera, beetles, and others, including acarus and nematodes.

Neem's oil formulations usually show a range of different azadirachtins amounts, ranging from 1000 to 4000 mg/kg, meaning that products can be obtained either by using directly poor neem oil or a dilution process of neem extracts containing different quantity of azadirachtins, up to 5%. In addition to neem oil, azadirachtins are also marketed, in particular azadirachtin A. The amount of production of this substance amounts to about 64 tons, with 80% coming from India, and China as the second producer. Other data about the activities of NSO and its products can be found in the references at the end of this chapter.

Larvicidal activity of neem

Our first experiments clearly demonstrated strong larvicidal activity of NSO and neem cake on Asian tiger mosquito (Nicoletti et al., 2012a,b). However, our HPLC and HPTLC analyses showed a low content in azadirachtins in the NSO and in the methanol extract (Mariani et al., 2013). The result was interesting, since it is well-known that insecticidal activity is strictly related to the chemical composition, but in contrast to most reports evidenced a relation between the activity and the presence of these limonoids. This consideration prompted research to identify a relationship between composition and activity in the case of NSOs marketed by different producers. First, the HPTLC analysis indicated great differences in the fingerprints of the analyzed oils, with special reference to limonoids (Nicoletti, 2011; Toniolo et al., 2014). A second analytical step consisted of a fractionation of three selected neem oils in three fractions of increasing polarities (i.e., ethyl acetate fraction (EA), butanol fraction (BU), and water (WE)). The initial neem oil and the obtained fractions were evaluated for larvicidal toxicity and field oviposition deterrence against the Asian tiger mosquito, *Aedes albopictus*. The experiments showed good toxicity of the entire neem oil and EA fractions against *A. albopictus* fourth instar larvae (with LC50 values ranging from 142.28 to 209.73 ppm), while little toxicity was exerted by BU and WE fractions. The differences of activity were in accordance with the results of HPTLC analyses, since the NSOs more concentrated proved to be more active. These results were confirmed by deterrence of *A. albopictus* oviposition in the field (effective repellence values ranging from 98.55% to 70.10%), while no effectiveness of BU fractions was found. Concerning ovideterrent activity, no difference due to the production site was found. These experimental data evidenced the possible use of neem constituents against Culicidae in the field. The constituents must be found in the apolar fraction, but the HPTLC analysis showed a complex composition, wherein limonoids were not prevalent. Therefore, neem oil and EA fraction seem promising, since they are effective at lower doses, if compared to synthetic products currently marketed, and could be advantageous alternatives to develop newer and safer mosquito control tools, but other studies are necessary to obtain a better definition of the active constituents and tailor the neem products in accordance with the required utilization (Benelli et al., 2015c; Mariani et al., 2013).

Therefore, when we started our work on neem products, we found several incongruities between the reported studies in the literature and our results (Nicoletti et al., 2017; Mariani et al., 2013). In case of incongruence of the experimental data, two main interpretations are possible. The anomalous data could be the consequence of some error in the experimental procedure or the previous reported data must be reconsidered on the light of the new ones. In fact, many scientific important discoveries have been as consequences of unexpected results. There is a strong tendency in pharmacology to assign the activity of a plant drug to one constituent, or eventually a few of the same chemical class. This is mainly a consequence of the pharmacological tests, which are tailored on the magic bullet axiom and the difficulties in determining precisely the composition of an extract. However, in an extract, and consequently in the plant, there are hundreds of compounds, with effects on bioavailability, solubility, and synergic and antagonist activity. In opposition to the magic bullet, there is the approach of the phytocomplex, invoked by many researchers in phytochemistry and pharmacognosy.

An important part of research on neem was dedicated to increase its availability and properties, focusing in particular on stability and cost, toward the production of the ideal insecticide. The first aspect was assigned to the production of nanobioparticles containing neem extracts, which demonstrated clear larvicidal and deterrent activity on vectors, like *Ae. aldopictus*, also in field conditions (Chandramohan et al., 2016; Murugan et al., 2016).

Several factors must be considered in the case of a product based on natural substances. In theory, the plant could be available for everyone and therefore it cannot be patented. Therefore, so far natural products are available for everyone and thus have been explored very little. Natural products are the chemical part of the environmental interactions between living organisms and therefore they are natural candidates for the production of active drugs. The chemical production of a plant is strictly subjected to the environmental conditions that can highly influence this production. First, the exactly determined species must be used and determined in composition. Once the raw material is obtained, the process of transformation can significantly influence the composition of the product. The technological transformation is essential to the quality and efficacy of the product. Therefore, this second step is vital for the success of the product. The third step consists of the target being assigned to the product and the consequent marketing.

In future, natural products will be even more important in the production of new drugs and foods and feeds, able to face the challenges of a continuously changing market. Technology is key to this. The natural products market is expanding rapidly in previously unexplored areas, in particular as an alternative to products based on synthetic compounds. The prospects and possibilities in this situation are immense, but knowledge of nature and activity of natural products must be revised utilizing recent devices and research approaches. Importance and role of natural products will increase if the multidrug resistance continues, asking for new bacterial and insect possibilities of control.

The common composition of a botanical product is based on a single herb or on the combination of more species based on recipes and formulae mainly derived from the historical literature and empirical experiences. The long and accurate work of phytochemistry based on the sequence extraction-separation-identification, derived from the correspondence of one drug to one illness, generated a huge catalogue of identified natural substances that can be employed as useful standards to determine the composition of the botanical drug. The knowledge about composition must be as complete as possible; not a single constituent should be unused, and utility depends strictly on the utilization.

Natural products are derived mainly from plants as the result of coevolution between organisms and environment (Tehri and Singh, 2013). For this reason, they have been used for centuries in popular and traditional medicines, as well as often being employed as spices and insecticides. Unlike modern pharmacology and drug development, which are based on a single chemical entity, natural product preparations are multiingredient. A single herbal drug contains at least 100 compounds making a complex matrix, named a phytocomplex, in which the single active constituent is not considered solely responsible for the overall efficacy. The utilization of the phytocomplex is based on experimental basis, since many data afford the validity of this approach, although further confirmations could be obtained using modern pharmacological devices. In other words, the same botanical raw material can be used directly, or extracted in different ways or used as a source of selected substances, or modified according to the product and target.

In 2010, a mixed team of experts from MIT (Massachusetts Institute of Technology) and the Broad Institute of Harvard University, both in the USA, reported an interesting and innovative study for a scientific evaluation of the effectiveness of natural products. The argument is strictly inherent to the endless debate about the role of natural products and their efficacy, causing a fighting contrast, but useless and boring, between supporters of "natural" versus defenders of synthetic drugs. The key aim of the study was to understand what is going on between the two main levels of the metabolism (primary and secondary), on the basis of the consideration of the functional connection between genes and gene products, as well as between genes and targets. An innovative feature of the study is that the researchers decided to commit the argument to neutral judgment, submitting the elaboration of collected data to the computational work of artificial intelligence. The work was based on the comparison of cumulative connectivity distribution of small molecules, natural or synthetic, grouped according to connectivity associated with the target. Assuming that proteins form biological networks and that metabolism and health depend on these networks, we should be able to assign a role to the molecules considered as possible medicines. The result showed that natural products target the proteins with a high number of protein-protein functional interactions (higher network connectivity), whereas the synthetic ones act on a limited protein network. The conclusions of the study, based on a computational approach, were evident: "We observe that approved drug targets that are not also natural product targets exhibit a connection distribution much closer to the case for human disease genes that natural product targets, which remain the most highly connected targets." This sentence indicates a positive and useful consideration about the role and activity of natural products. Natural products tend to target more essential and general protein networks to an organism than other groups of small-molecule targets, like those more related to specific disease genes. Therefore, the dichotomy between natural and synthetic active constituents must be considered mainly as a consequence of a cultural heritage, unable to assign a complementary or differently appropriate role to the two classes of molecules. The results of the study are coherent with the nature of natural products, whose production is the consequence of environmental interactions, including defense against predators or pathogens. This kind of defense cannot be specific, and therefore natural products act on more highly connected network of proteins, interrupting or limiting the activity of the essential proteins in environmental competitors or invaders. They may be tailored for a positive or negative influence in physiologic activities and basic metabolism of an ample range of organic targets. These arguments are in favor of the potential use of natural products as insecticides.

In any case, there are several difficulties in assigning the activity to single constituents, causing several cases of wrong or misleading assignments of all the activity to single substances in the case of a plant extract, like in valerian (*Valeriana officinalis*), whose extracts are largely marketed and utilized for their mild sedative effects. With the discovery of valepotriates, the effects were assigned to these constituents, but after the evidence that extracts with low content in instable valepotriates also exerted similar action, the essential oil was considered additionally responsible for the effect. Another case consists of a current debate about hemp. Besides cannabinoids, its essential oil and other constituents are now considered important for the multiple activities of hemp. In other words, there are hundreds of marketed products of hemp and many related claimed activities, and this can be related to the complex cannabidioma and/or the different compositions of the products, although they are all derived from the same raw material. It is very important to stress that important new features can appear, also in the case of species highly studied in their chemical composition, as shown in the scientific literature. Recently, a new cannabinoid was isolated from *Cannabis sativa* (Citti, 2019). As is well-known, (−)-*Trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main compound of hemp and it is considered the main one responsible for intoxicant activity. However, the chemical constitution of this species is subject to high differences in accordance with its varieties and cultivars. Cannabinoids possess a unique structure, derived by junction of a monoterpene and a polyketide unit. Most of them have a side alkyl chain, whose length influences the biological activity of this cannabinoid. In fact, analogues of Δ^9-THC with a longer side chain were synthetized and they have shown cannabimimetic properties far higher than Δ^9-THC itself (seven C against five). In this study, a new phytocannabinoid with the same structure of Δ^9-THC, but with a seven-term alkyl side chain, was isolated and identified, and its stereochemical configuration confirmed by a stereoselective synthesis. This new phytocannabinoid has been called (-)-*trans*-Δ^9-tetrahydrocannabiphorol (Δ^9-THCP). The binding activity of Δ^9-THCP against human CB_1 receptor in vitro ($K_i = 1.2\,nM$) proved to be similar to that of CP55940 ($K_i = 0.9\,nM$), a potent full CB_1 agonist. In the cannabinoid tetrad pharmacological test, Δ^9-THCP induced hypomotility, analgesia, catalepsy, and decreased rectal temperature, indicating a THC-like cannabimimetic activity. As confirmation, the corresponding cannabidiol (CBD) homolog with a seven-term side alkyl chain (CBDP) was also isolated and unambiguously identified by matching with its synthetic counterpart. The presence of this new phytocannabinoid could account for the pharmacological properties of some cannabis varieties that are difficult to explain by the presence of the sole Δ^9-THC and indicate the importance of the interaction between constituents of the so-called cannabidiome.

Therefore, we were not totally surprised when we found good larvicidal activity against *Aedes albopictus* also in NSOs with low content in azadirachtins (Mariani and Nicoletti, 2013). This was quite a novelty on the basis of the literature, but it is necessary to consider the importance of the metabolomics approach and the possibility with HPTLC to obtain several views of the same plates. Each view means a revelation of different compounds on the basis of their chemical structure and present functional groups. Using an appropriate revelation agent, it is possible to see compounds that are not visible with another derivatization. This approach is contrary to the tendency of current analytical chemistry to focus on a single class of compounds or even unique constituents, which obtain perfect and reliable but limited results. Another incongruence consisted of the presence of insecticide activity also in neem products after years of production, when limonoids should be highly degradated.

Neem's mechanism of action

The first experimental evidence we obtained on the activity of neem oil was the inability of the larvae of *Ae. albopictus* to complete the molt from larva to pupa. The larvae proved to be initially immature, their bodies imperfect, and finally before the third instar, most insects died and none was able to fly. The delicate mechanism of the development stage was jammed and the cruel destiny of the unfortunate insects assigned. Each organism has its weakness. Mosquitos, like any arthropod, possess a rigid exoskeleton, which offers efficient strong and secure protection, also against pesticides, which is one of the reasons for the success of these creatures.

The exoskeleton of insects is primarily made of proteins (sclerotin) and chitin (a polysaccharide), which are interwoven and linked together to form strong but flexible bundles. Interestingly, chitin is also the main constituent of the fungal cell wall. The ratio of the components of the exoskeleton varies from one body part to another on an insect. However, the exoskeleton is too rigid, and acts like a cover that encases the entire insect, and being a non-living formation, the exoskeleton does not change size and grow with the insect. The exoskeleton is too ridged to be recycled or modified, and it must be substituted, but it must also protect the insect until the new exoskeleton is ready. During the growth period, insects must shed the exoskeleton in order to assume a new form. As a result, it is necessary for the insect to shed its old exoskeleton to make way for a new, larger one through a process called molting. This is a hormone-controlled phenomenon. During the molting stages, the hormones are released to start and finalize each step of the metamorphosis, until the mature insect finally emerges. However, the chemical constitution of the exoskeleton is variable in each insect species and this is the reason for the selective toxic effects, such as those reported in the case of neem. Regarding the structures of insecticides acting as growth regulators, albeit in the case of ecdysones the relation with insect hormones is evident, in other cases the similarity is not so clear, as well as the real mechanism of action.

The stages between the subsequent molts are generally called instars. These correspond to altered body proportions, colors, patterns, and changes in the number of body segments or head width. For most insect species, an instar is the developmental stage of the larval forms, but an instar can be any developmental stage including pupa or imago. The larval stage is in particular a delicate stage of the insect metamorphosis.

However, we were totally aware that confirmation of the neem insecticide activity, albeit with a demonstrated chemical constitution, in a laboratory experiment was a weak starting point. The open questions were numerous: (a) how to obtain the same result in the field; (b) whether the larvicidal activity could be connected to other properties, in order to improve the use; (c) what the cost of neem oil would be, considering the large-scale spread of the insects; (d) how limited the stability of the active ingredients of neem would be; (e) what determination of chemical content of neem oil would be required, to be connected to the determination of the activity; and (f) what the ambit of utilization would be and the possible damage to the habitat.

Other advantages arising from the use of neem-based products are the rare induction of resistance, due to their multiple mode of action against pests, the low toxicity rates that have been detected against vertebrates, and finally the necessary environmental care.

The alternative to neem

There is a little confusion about the plant species named azedarach, and very similar denominations. The name azedarach was given by the famous Persian physician Avicenna (980–1037) to indicate some poisonous trees; however, *Azadirakhti* literally means "free book of India." In 1753, Linnaeus reported about *Melia azadarachta* in his *Species Plantarum* (1: 385 with habitat: India). In the same book (1: 384), we can find *Melia azedarach* (habitat: Syria) and *Melia azedarach* var. *sempervirens* (habitat: Zeylona). Actually there are two distinct species, *Azadirachta indica* A. Juss, attributed to neem (or nimba, meaning "who gives good health," as reported in the Sanskrit books) and *Melia azedaracht* Linneus, attributed to melia, a very similar tree. This is the typical taxonomic situation in botany and zoology. The differences between taxa are often very narrow and only specialists are able to find them. In any case, the problem of the significance of these differences is always a matter of debate. God bless taxonomists, because they are necessary to obtain order out chaos, but please do not spend your precious intellect on endless discussions with no final consistent result! In fact, the matter is complicated by synonymous, parental disputes, errors of any type, including wrong transliteration (i.e., gingko and ginko), disputable rules of the international codices, and more.

Neem and melia are very similar, but there are several tricks to distinguish between the two species. The first is commonly known also as Indian lilac and the second one as Persian lilac or simply melia. Neem has usually white flowers whereas melia presents an explosion of blue flowers; the fruits of the former have an elongated shape, whereas the latter's are totally rounded. If the trees do not have flowers or fruits, and you are not a botanist, you may be in trouble, but you can remember that neem cannot live in temperate climate regions, whereas melia can be easily cultivated in such places. Therefore, if you are in Europe or the USA, you can be 90% sure on the matter.

Melia azedarach is known by several common names, such as melia, chinaberry tree, Pride of India, bead-tree, Cape lilac, Syringa berrytree, Persian lilac, and others. It is usually a large tree growing up to 30 m tall, with leaves 2-pinnate, rarely 3-, with primary pinnae in two to six pairs, usually three to seven leaflets per pinna, narrowly ovate or subovate, serrate, acuminate, irregularly toothed, or crenate. Flowers are abundant and small, sweet-scented, in large axillary panicles. All parts of this tree are reported to have medicinal uses, but in particular, in terms of insecticide properties, seedlings are reported to present aphid attacks. A leaf used as a bookmark will deter insect pests. In Italy, the tree is known as the tree of rosary, since in the past, before the advent of plastic, its hard and round kernels were used to make the grains of a rosary.

Our research on *Melia azedarach*, as well as the references on this plant, evidences a significant difference in the chemical composition. Limonoids are present, but different from azadirachtins and other constituents make a marked relevant dissimilarity in composition. The initial conclusion was that melia probably cannot compete with neem as an insecticide, but other utilizations can be explored. However, once again a limit in the references is an irresistible task for a researcher in search of innovations.

In addition to the insecticidal properties, we were initially particularly interested in the antimicrobial activity. People often associate antimicrobial activity with infection and effects on their health, but microbes are everywhere and most damage affects cultivation of plants. Agricultural methods of reproduction of plants with economic value were totally transformed by the introduction of micropropagation and stem cell culture. Micropropagation allows the rapid cultivation of selected cultivars, saving time and resources. However, although the first steps of micropropagation were performed in aseptic conditions, the possibility of infection of calla, shoots, and seedlings is high. Avoiding the infection must be done via an appropriate and sensitive approach, avoiding damage to the delicate meristems—a typical job for natural products.

The antibacterial study (Marino et al., 2014) aimed to investigate the antibacterial activity of unripe fruits of *Melia azedarach* collected in different periods. The activity was tested on the shoots of a hybrid of *Prunus cerasifera* x *Prunus spinosa* and calla lily of *Zantedeschia aethiopica* against several bacterial species. The data reported evidenced a positive antibacterial activity and the absence of any negative effect on the growth of shoots surviving at the second subculture on a standard medium. HPTLC analysis showed the prevalence of polyphenols, such as chlorogenic and caffeic acids, which, on the basis of the literature, are consistent with the antimicrobial activity. This activity is important considering that many plant species of economic relevance are now obtained by micropropagation, and this cultivation in vitro is necessary to avoid any sort of contamination.

Further research is essentially the rational collection of most of the arguments previously considered, as evident in the title: "Green-synthesised nanoparticles from *Melia azedarach* seeds and the cyclopoid crustacean *Cyclops vernalis*: an eco-friendly route to control the malaria vector *Anopheles stephensi?*" (Anbu et al., 2017). In this research, once a single-step green-synthesis of silver nanoparticles (AgNP) using the seed extract of *M. azedarach* was obtained, we tested its mosquitocidal activity. In laboratory assays on *Anopheles stephensi*, Ag NP showed LC_{50} ranging from 2.897 (I instar larvae) to 14.548 ppm (pupae). In the field, the application of Ag NP ($10 \times LC_{50}$) led to complete elimination of larval populations after 72 h. Finally, we decided to test the nanoparticles on nontarget aquatic predators. The application of Ag NP in the aquatic environment did not show negative adverse effects on predatory efficiency of the mosquito natural enemy *Cyclops vernalis*. The reason for this additional research lies in the fact that numerous aquatic arthropods attack and devour preparasites. As we already know, the utilization of the insecticides, though with plant-derived active constituents, could be dangerous for the environmental equilibria. In particular, it could affect the natural biological control, based on the presence predators of the vector in the common habitat, remembering that all the insect stages, except the adult insect, need water. In such sites, there is fresh water everywhere, such as lakes, pools, and similar places, enabling life along the plant-covered banks of stagnant and slow-flowing bodies of water. In such places, mosquitos can proliferate as can any other predator, which in an aquatic environment is fundamental to limit the proliferation of the vector. In fact, after coupling, and the consequent blood feeding necessary to assume the proteins necessary for the eggs maturation, the female is looking for an appropriate place for the deposition of 100–500 eggs. A single *Anopheles*, like other insect, is able to produce a quantity of eggs and larvae enough to invade all the neighboring habitats, as in the classic case of a locust invasion. This is not possible only thanks to the natural enemies. The micro-aquatic environments are the scenario of a continuous fight for survival, where often two or more species of arthropods are involved, as predator or as prey. In our study, we selected the genus *Cyclops*, which is one of the most common of freshwater copepods, comprising more than 400 species. Copepods are very little crustaceans, commonly called water fleas. They have a single large eye, which may be either red or black, and therefore they are named for the Cyclops of Greek mythology. *Cyclops* prefers fresh water, and is less frequent in brackish water, where it feeds on small fragments of plant material, animals, or carrion. It swims with characteristic jerky movements and has the capacity to survive unsuitable conditions by forming a cloak of slime, with an average lifespan of about 3 months.

Several microscopic crustaceous, including copepod species, feed small and very small preys. In high-density, unstructured environments such as eutrophic lakes, predatory copepods commonly coexist with certain small-bodied prey, where encounters are frequent with ineptness on the part of the predator and counter-tactics by the prey. In particular, laboratory studies showed that copepods are effective predators on early-instar *Culex* larvae, involving an important role in suppressing mosquito populations, because of their feeding behavior and abundance. They are very efficient in this role, since the presence of alternative abundant food, like bacteria and protozoa, does not deter their attacks on their preferred prey. Copepods are capable of killing and eating at least four preparasites within 13 min and a predator density of 53 copepods/liter is expected to reduce the mosquito larvae by 50%, with the rate of predation inversely proportional to the water volume.

Neem cake: From by-product of an industrial process to multipurpose resource for a sustainable agriculture chain

During our research activity, we were highly interested in industrial plant-borne by-products, since they can offer new products to the market with lower cost and high usefulness. Our attention was immediately attracted by neem cake, a cheap by-product of NSO extraction, obtained as a residue after mechanical pressing of the neem seeds, considered of low economic value and utilized to enrich the soil of some mineral components, such as nitrogen.

The laboratory test indicated neem cake activity against *Aedes albopictus* and a number of *Culicidae* species (Nicoletti et al., 2010). In the case of biocidal treatments, it is important to demonstrate that insecticide activity is associated with antimicrobial activities in consideration of the high possibility of infection and the severe consequences for health, in particular in the case of the animals, both pets and livestock. The importance of insecticidal and antimicrobial activities for animal treatment has been evidenced in the experiments with NSO and neem cake further reported, including larvicide, deterrent, and repellent activities (Benelli et al., 2015a,b; Mariani and Nicoletti, 2013; Nicoletti et al., 2012a,b).

The complex range of different compounds in the neem seeds open the possibility to utilize the derived products to solve many current problems. The challenge now is to obtain marketed products tailored for different utilizations. The reported experiments evidenced these potentialities, which are only waiting a realization and a wider utilization.

Despite diseases, wars, and environmental disasters, the human population is growing. First of all, more people will need more food. This forecast shows in particular a massive increase in animal protein demand, needed to satisfy the growth in the human population, wherein billions of people require an increase of caloric input and better food. Therefore, attention is focused on the sources of feed protein and their suitability, quality, and safety for future supply. In addition, the quantitative production aspect is causing a series of problems. There will need to be a considerable increase in feed manufacture, requiring a thriving, successful, and modern feed industry, including a key aspect concerning the protection and preservation of the food produced and marketed. This aspect is strictly related to safety issues, which will remain paramount in the minds of consumers following recent food crises.

It is time to consider that the need of more food to feed due to increasing planet population perhaps cannot simply be solved by massive production, but reduction of food waste and conservation can increase food availability by 30%–40%. “Feed the Planet and Energy for Life” was the theme of World Expo 2015 in Milan, Italy. Among the activities occurring at the Expo, research and proposals concerning the utilization of neem products were presented in a call for projects in favor of sustainable progress and production of future foods. The Neem Project was selected as the best one due to its possible applications in the production of food and feed.

The Expo event projected feeding as the main challenge for humankind and showed the extreme urgency of elements of innovation in technology and science connected to the production and conservation of food. It was demonstrated how serious feed problems still plague several areas of the world today, and the possibility of new solutions was mentioned. The Neem Project was focused on the agricultural utilizations of neem cake concerning its advantages as soil fertilizer and as a natural ectoparasiticide for the treatment of sheep and goats.

Neem products were proposed as being able to affect the biotic composition of the soil. Neem cake must be preferred to neem oil for its cost and its form as a powder immediately available. Several experiments evidenced the improvements of the utilization of neem cake in agrarian ecosystems:

(1) availability of nutrients, in particular nitrogen and phosphorus, more consistent with the requirements of the crop;

(2) development of the microbial beneficial biomass of the soil, which increases in quantity and activity, but with selective influence against nematodes and other negative components. In agricultural practices, plants in addition to nutrients should count on a greater variety of useful microorganisms and on acquisition of nutrients themselves, through the activation of complex symbiotic systems. If you want to understand the state of health of a tree, you must look down, not up; and

(3) development of pest control system of insects and other arthropods of agricultural and livestock interest.

Neem cake, as an industrial by-product, is a heterogeneous material that maintains a high added value due in large part to its chemical composition, which confers its biological activity. Neem cake is widely available on the global market, considering the increasing presence of neem trees in the world and production of NSO. The exploitation of its characteristics in the food chain to improve consumer health, increase the productivity of agricultural products, and feed the planet is the logical consequence of the urgent need to develop new sustainable agricultural systems in a world where many highly polluting pesticides are no longer allowed to be used. However, more research, in particular in field conditions, is necessary to understand the real value of its microbiological, insecticide, fertilizer, and nematocide activity, involving collaborations between different experts in individual sectors—import companies, organic farms, and research institutions—in order to determine the manner and timing of land application of this valuable product of "waste," still underestimated.

Neem cake could lead to a revolutionary improvement in the fertilization of agricultural plants, adding to the characteristics of chemical fertilizers those of a soil improver. In agriculture, we could define neem cake as a prompt nutrient-release fertilizer, effective in allowing rapid absorption of nutrients and promoting development of the plant, with the capacity to increase the activity of the microbial biomass and organic matter, favoring the sequestration of carbon. The idea was to join the fertilizer, insecticide, and antimicrobial utilities of neem cake.

Exploitation of the use of neem cake as an insecticide came from this first test: some pots of impatiens (*Impatiens balsamina*) were fertilized with 3% by volume of neem cake, and 500 mosquito larvae were reared starting from eggs. The eggs were hatched in control and treated in pot saucers, but none of the newborn larvae survived in the water saucers of pots treated with neem cake, while in the water saucers of pots unfertilized with cake, the 500 control larvae completed their development in less than a week, becoming adult mosquitoes. Other major beneficiaries of the use of neem cake as insecticide are undoubtedly sheep farmers, who can use an organic product of natural origin and low cost that is simultaneously effective against the larvae of Culicoides and other pests, while respecting the natural biotic communities.

Direct beneficiaries of neem cake, as a fertilizer, are farmers seeking pest and nematode control, in particular for nematodes. Currently, some highly toxic products are still on the market by virtue of the absence of suitable alternatives. Particular attention must be focused on the changes on soil micro-composition, as evidenced in several field experiments.

In conclusion, we can report the following important advances in the use of neem cake as a functional fertilizer:

(a) energy saving flows from the use of a waste of an industrial chain;

(b) environmental sustainability, as documented by the analyses attesting the absence of heavy metals, aflatoxins and residues of pesticides;

(c) neem cake is an excellent alternative to methyl bromide (BM) (banned as being responsible for the "thinning" of the ozone layer of the atmosphere);

(d) neem cake is an excellent alternative to Temephos and other organophosphates used to treat water infested with disease-carrying insects including mosquitoes, midges, and blackfly larvae;

(e) neem cake is a great alternative to nematicides, like 1,3-dichloropropene; and

(f) neem cake in the field trials carried out in Sardinia had efficacy similar to azadirachtin biological products already established in organic farming, but were very expensive and not really effective. In addition, neem cake showed very low effect on "nontarget" insects that live in the same environments as Culicoides larvae.

Neem oil as an antibacterial natural product in pest control in livestock

Ectoparasites are organisms that inhabit the skin of another organism, causing significant infestations and pathologies. Many micropathogens can profit from the work of ectoparasites, either to colonize the skin injury and lesion, or be inserted in the host during the feeding. The vast majority of ectoparasites are arthropods, e.g., insects and arachnids. Again the triangle host-vector-etiological agent is reproduced.

Many ectoparasites are vectors of pathogens, which are typically transmitted while feeding on or from other hosts. Several ectoparasites (e.g., most lice) are host-specific, including livestock, pets, poultry, fish, and bees, but others parasitize a wide range of hosts, including humans. Typical effects of infection on the host are irritability, dermatitis, secondary infection (other parasites profit of the skin necrosis), fecal hemorrhages, blockage of orifices, inoculation of toxins, and exsanguination. As a consequence, the host's general health can be seriously affected with low weight gains, particularly important in livestock. Subdermally located parasitic larval stages of certain flies can be favored by the ectoparasited infection, causing a condition termed "myiasis." When insects (order Hemiptera) are involved, the infection mechanism is similar to that previously described for any insect-borne disease. The vector contains several hematophagous ectoparasites, including approximately 150 species of kissing ("cone-nose") bugs (Reduviidae, Triatominae) and bed bugs and bat bugs

(Cimicidae). These parasites make physical contact with the host principally when ingesting a blood meal. These kissing bugs usually prefer domestic animals, from which relatively large blood volumes may be imbibed; in such a way they can cause a great deal of damage and transmit important diseases.

Ectoparasites play a very detrimental role in terms of decreasing the productivity of livestock, such as sheep and goats. NSO was utilized in the field as an antibacterial in the case of ectoparasites' stings and bites resulting from goat wounds. Common external sheep and goat parasites include ticks, lice, and mites. They cause restlessness and irritation. Weight loss and reduction in milk production may occur as a result of nervousness and improper nutrition, because animals spend less time eating. Bites can damage sensitive areas of skin (teats, vagina, eyes, etc.). Some parasites feed on blood, causing anemia, especially in young animals. The bite and the sting of ectoparasites allow bacteria to proliferate in wounds from abrasions or lesions from scratching, and cause levels of tissue reaction of different entities, super-infection, and cervical lymphadenopathy.

Ectoparasites cause many problems in livestock production. They seriously damage sheep and goat skins, resulting in the rejection or downgrading of the skins. This causes huge economic loss, as this skin damage renders it unsuitable for the leather industry due to the decrease in quality. Lower production of meat is also a typical consequence. *Pseudomonas aeruginosa* wound infections are characterized by a change in the color of the skin around the wound area and the formation of lesions. The bacterium products and pigment cause yellow discoloration of wool and consequently reduced quality and market value.

NSO treatment in the field on as a natural ectoparasiticide for sheep and goats proved to be successful in preventing and curing the attacks of endoparasites (Fig. 7.16). The experiments were performed on selected livestock (Fig. 7.17) by a specialized team of CREA researchers (De Matteis et al., 2015). The effects on the parasites were evident (Figs. 7.18 and 7.19) and even after the first treatment with NSO, protection against ectoparasites was obtained. More important, the health of the treated livestock improved, as testified by the hematological profile of goats. *In vivo* and in vitro tests on blood cells from Siriana, Sanen, Cashmere, and Maltese goat (*Capra hircus*) breeds showed no significant difference ($P < .05$) between NSO treated and untreated goat hematological parameters at each sampling time considered. In addition, the NSO effect on goat PBMC cultured in RPMI medium was evaluated at $1{:}2 \times 102$ to $1{:}20 \times 106$ dilutions at 14, 21, and 40 h of exposure. The in vitro test revealed that the response of goat PBMC viability is

Fig. 7.16 The preparation neem oil solution for the experiment in field.

Fig. 7.17 The preparation of the ovine.

Fig. 7.18 Effect of NSO solution on the parasite (*Linognathus stenopsis*).

Fig. 7.19 Effect of NSO solution on another parasite (*Damalina caprae*).

dependent on concentration, incubation time, and NSO dose. In conclusion, the NSO should be considered useful, safe, and innovative for development of topical solutions for the care of wounds.

Among the most relevant typology of neem products, we focused on selectivity. The antibacterial activity of NSO was assayed (Del Serrone et al., 2015) against 48 isolates of *Escherichia coli*, considering that this bacterium can produce beneficial and pathologic populations. The molecular biology characterization showed that 14 isolates resulted in diarrheagenic *E. coli*. NSO showed biological activity against all isolates. However, there were significant differences between the antibacterial activities against pathogenic and nonpathogenic *E. coli*, as well as between NSO and ciprofloxacin activities. On the basis of the results obtained, NSO is able to counteract *E. coli* and also influence the virulence of *E. coli*-viable cells after treatment with NSO.

Saving food to save lives

The preservation of marketed food is an important aspect of the smart utilization of the produced food (Maruchecka et al., 2011). Furthermore, the consequent waste of unutilized food is a relevant problem in over-crowded towns (HLPE, 2014). A large quantity of food is lost or wasted throughout the supply chain, from initial agricultural production down to final household consumption (HLPE, 2014; Kader, 2005). The loss or waste for high perishable food, such as fresh fruit and vegetables, fish and livestock products, has been estimated at as much as half of all food grown before and after it reaches the consumer. Approximately one-third of all FFVs produced worldwide are lost during food supply chain production. Shelf life plays a central role in food spoilage. The impact of the enormous quantity of packaging is evident in any planet environments. Increase of the shelf life means reduction of cost and waste. Everything pivots around the material utilized for packaging, and new solutions are emerging (Otoni et al., 2016; Singh and Singh, 2005; Cooksey, 2005; Appendini and Hotchkiss, 2002), including passive packaging (Brockgreitens and Abbas, 2016; Ozdemir and Floros, 2004), active packaging (Coma, 2008), intelligent packaging (Lee et al., 2015; De Kruijf et al., 2002), and smart packaging (Dobrucka and Cierpiszewski, 2014). Although the results are not evident in our ordinary life, the galaxy of packaging is rapidly moving and increasing in research and proposals, based on new technologies and advanced techniques recently available, like nanotechnology and molecular biology. Efforts are focused on solving the food preservative problems, to extend the shelf life of perishable foods, by reducing the need for additives and preservatives.

"Smart packaging" is based on the production of functional methods to obtain the following goals: be tailored depending on the product being packaged, including several types of food, beverages, pharmaceuticals, household products, etc.; reduce food waste, increasing the shelf life; and maintain, and eventually enhance, product attributes (e.g., look, taste, flavor, aroma). The key words are protect, preserve, and present.

Several methods and approaches, such as oxygen scavenging and antimicrobial technologies associated to the production of modified films, have been considered (Del Serrone and Nicoletti, 2014). They are different solutions to serve the basic and fundamental properties of packaging. So far, the dominant packaging is the basic one, using low-cost material and involving no interaction with the food inside. This is passive packaging, wherein the traditional packaging systems are included, as the use of covering material characterized by some inherent insulating, protective, or ease-of-handling qualities. Usually, the ordinary packaging of food is mainly a used to method to attract and select the consumer, beside a preservation. The consequence is the enormous amount of waste, and the consequent damage to the environment. This situation is increasing due to the increasing numbers of consumers in emerging countries, where these consequences are not adequately considered. Packaging is considered active when it can interact in the same way and/or react to various stimuli, in order to keep the internal environment favorable for the maintenance of product quality. Several environmental, biotic, and abiotic factors must be considered, in order to respond to the degradation process successfully. The activity involved could be the presence of an oxygen scavenger (this can absorb high-energy oxygen inside a package and therefore increase the shelf life of a product) or an anti-ROS (a scavenger of radicals by oxygen or other origins), such as in the typical case of phenolic natural products. Smart packaging relies on the use of chemicals, electrical, electronic, or mechanical technology, or any combination of these. Technology is used to modify the packaging by adding constituents to change its features and properties (Kerry et al., 2006; Malhotra et al., 2015). Active and intelligent packaging is particularly dedicated to the preservation of fresh products, like vegetables, in accordance with increasing requirements for this kind of food (Nicoletti and Del Serrone, 2017; Nicoletti, 2014a,b). Intelligent packaging systems monitor the condition of packaged foods to give information regarding the quality of the packaged food during transport and storage (Aguilera et al., 2003). Probably the most innovative aspect of intelligent packaging is that it can be supported by the utilization of systems of detection in meat and meat products, obtained through the use of sensor technologies indicators (Thakur and Ragavan, 2013), including integrity, freshness, and time-temperature indicators (TTIs) and radio frequency identification (RFID). Therefore, active and smart packaging performs additional functions to the basic one by the introduction of innovations in the design of packaging, with the aim of increase the shelf life, but also to add conveniences for the user and usefulness for the consumer, to be introduced also in the supply chain. In this way, the product can respond not only to the need for a longer life, but also make the product more available, more useful, and more safe.

Since our invisible enemies are asked to play their role again, antibiotic activity is required. Packaging is mainly used to separate food from environmental conditions, utilizing simple material made of paper or plastic. However, it cannot prevent internal attacks by microorganisms, but can only limit or delay the effects. Therefore, additional treatments are required to limit their action, like the utilization of low temperatures, which involves additional costs and energy consumption and pollution. A new idea is to associate to the packaging some antimicrobial agent. Before and during packaging, storage, and shelf life, food is subjected to a continuous attack by microorganisms. These microorganisms are working to benefit themselves by demolishing progressively the molecular structure of the food, as soon and as completely as possible. Therefore, by preserving the food, we are working in a thermodynamically unfavorable situation. In term of shelf life, the food is in competition with its natural recycling, and, working to maintain as possible this limbo, we can utilize the food efficiently as it is possible.

The resistance phenomenon interests also zoonotic food- and water-borne pathogens becoming more resistant to antibiotics (Del Serrone et al., 2006). Resistant strains of pathogens have been isolated from food, causing an increasing incidence of food-borne diseases. Through the food, these microorganisms could be entering the human gastrointestinal tract on an almost daily basis. The antimicrobial activity of NSO and related products have already been reported (Palanappian and Holley, 2010; Baswa et al., 2001; SaiRam et al., 2002). A possible utilization of antibacterial activity of neem cake against meat spoilage bacteria was tested using a broth model meat system (Del Serrone and Nicoletti, 2013). The tests were positive, since the growth inhibition zone (mm) varied significantly ($P \geq .05$). With respect to ciprofloxacin activity, the antibiotic value ranged as follows: 11.33 ± 0.58 to 22.67 ± 0.58 mm and 23.41 ± 1.00 to 32.67 ± 2.89 mm, respectively. The percentage of bacterial growth reduction (GR%) also varied significantly ($P \geq .05$) in function of considered NCE concentrations (1:10–1:100,000), with the highest GR% for 10 μg NCE (79.75 ± 1.53 to 90.73 ± 1.53). The numbers of viable bacterial cells never significantly ($P \leq .05$) exceeded inocula concentrations used to contaminate the meat. All the results of the experiments showed that neem cake is able to counteract the main microorganisms responsible of meat spoilage, like strains of Gram-positive and Gram-negative, as well as facultative anaerobic bacteria. The antimicrobial activity of neem products was confirmed also for NSO against spoilage bacteria, such as *Carnobacterium maltaromaticum*, *Brochothrix thermosphacta*, *Escherichia coli*, *Pseudomonas fluorescens*, *Lactobacillus curvatus*, and *L. sakei*. After the second day after NSO, only *C. maltaromaticum*-viable bacterial cells were detected.

These data could be used to create new intelligent packaging. Utilizing a nanotechnology already employed for other materials, neem cake may be incorporated into the cavities of nanoparticles, maintaining its antiparasite activity. Once incorporated into the packaging material, the neem cake, also in minimum quantity, should be able to effect its preservative food action, acting against the demolishing microorganisms. The increase of the shelf life of meat should compensate for the additional cost of the packaging material, not considering the decrease of waste. It is possible that the first activity of LUCA was to find the energetic source for survival, and the second was to compete with the other LUCAs. The results are an endless transformation of forms and production of new molecules. The living organisms had a long time to organize their molecular weapons and the secondary metabolites are there, produced and organized to be considered and utilized in the right way. The neem tree is an example of nature's treasure. The advent of *Homo sapiens* (Lucy) changed in part the rules of the natural game, but natural products still remain a necessity for our life.

Malagasy plants as sources of chloroquine-potentiating agents

Recently, parenteral artesunate has replaced quinine and many other antimalarial products for the treatment of severe malaria. However, several reports have demonstrated the emergence of resistance to the efficacy of artemisinin-based combination therapy monotherapy, such as in western Cambodia and other regions in South-East Asia. To face the phenomenon, artemisinin-based combination therapies are now recommended by the WHO. The aim is to reduce the morbidity and mortality associated with malaria with artemisinin-based combination therapies, including chloroquine plus other drugs, like sulfadoxine-pyrimethamine. Meanwhile, with increasing resistance to chloroquine, quinine is reconsidered, being so far the only substance for which *Plasmodium* did not develop resistance. The consequences are that in Uganda quinine was prescribed for up to 90% of children younger than 5 years with uncomplicated malaria, and from 2009, 31 African countries recommended quinine as a second-line treatment for uncomplicated malaria, 38 as a first-line treatment for severe malaria, and 32 for treatment of malaria in the first trimester of pregnancy. Recent surveillance data from other sites are in accordance. However, quinine was substituted due to its limits and therefore in 2010, WHO (2010) guidelines recommended reinforcing quinine's activity by combining it with other antimalarial agents, like doxycycline, tetracycline, or clindamycin as a second-line treatment for uncomplicated malaria (to be used when the first-line drug fails or is not available) or quinine plus clindamycin for treatment of malaria in uncomplicated cases and in the first trimester of pregnancy. The development of effective cocktails is a current trend of medical treatment of several diseases, including forms of cancer. In addition, the combination of natural products and synthetic drugs is recommended.

Natural products can be utilized as resistance-modifiers or chemosensitizers, and may be able to restore chloroquine sensitivity in resistant strains of *Plasmodium*. The idea of 8 years of research from different research groups was that the antimalarial treatment combined with natural products could be based on lower doses of chloroquine, in order to minimize the resistance insurgence and to avoid the collateral effects in the case of prolonged use, necessary in areas where the disease is endemic. This approach came from an ethnopharmacological investigation by Professor Rasoanaivo (Rasoanaivo et al., 1992). Most people consider ethnopharmacology to be a collection of ancient utilizations of natural sources, and as knowledge that is going to disappear. On the contrary, in addition to traditional uses there are new ones emerging, even as consequences of the utilization of modern drugs. Considering that OMS reports that 80% of the planet population relies on traditional medicine, the utilization of medicinal plants is not limited to ancient times and past populations, but it changes according to needs and evolution of treatments. Ethnobotanical knowledge is still passed from one generation to another in the majority of populations living in rural areas, and in urban areas, where malaria has been revealed to be resistant or incurable by modern scientific medicines, people have turned to traditional treatments. It is therefore of paramount importance to preserve and transmit this ethnobotanical heritage. Therefore, this discipline must be regarded as a multidisciplinary science in movement, where botany, chemistry, and pharmacology play central roles for scientific evaluation and validation of popular uses. However, economic and social aspects must also be considered, in order to develop new drugs and treatments of both old and new diseases. Most antimalarial drugs currently in use belong to the classes of aminoquinolines (chloroquine, amodiaquine, primaquine), quimolinomethanol derivatives (quinine, mefloquine, halofantrine), diaminopyrimidines (pyrimethamine), sulfonamides (sulfadoxine, sulfadiazine), biguanides (proguanil and derivatives), antibiotics (tetracyclines, doxycyclin, clindamycin), sesquiterpenes (artemisinin, dihydroartemisinin, arteether, artemether, artesunate), and naphtoquinones (atovaquone). Among them, only quinine and artemisinins are natural products, but also a relevant part of the current antimalarial arsenal. The potentiality of natural products is very high. A review by Willcox and Bodeker (2004) on traditional herbal medicines for malaria in three continents reported 1277 plant species from 160 families. However, the clinical trials are largely lacking, since only eight clinically controlled trials have been reported, involving *P. falciparum* and *P. vivax*.

In the case of malaria, alkaloids are the first candidates responsible for the activity. There is a long tradition in popular medicine of plants containing these compounds to control fever. These plants also have a bitter taste, which is usually connected to the alkaloid presence, as already reported for the aforementioned quinine bark case.

Two important considerations attracted our attention, in view of the possibility to explore new strategies: the special endemic flora of Madagascar and the occurrence of information about a popular treatment of malaria as yet unreported. Madagascar is a land of endemism, consisting about 13,000 species of vascular plants, of which 80% are endemic, and eight families totally endemic.

Malaria is practically endemic in all Madagascar and therefore the population harbors a very rich and unique knowledge on antimalarial plants. After a resurgence of malaria in the early 1980s, as a consequence of *Plasmodium falciparum* resistance and due to the high costs of conventional drugs, local populations returned to the uses of herbal remedies. Two hundred and thirty-nine plant species, of which about 30% are endemic to Madagascar, have been reported as having antimalarial uses in Malagasy traditional medicine. Prof. Rasoanaivo discovered the use by some populations in Madagascar of decoctions of some local plants in association with low doses of chloroquine to complement chloroquine action against chronic malaria (Nicoletti et al., 2018). The lower use, one or two tablets of chloroquine (100–200 mg), is probably adopted to avoid collateral effects due to prolonged use of chloroquine, but such a dose could be considered inadequate to favor chloroquine resistance. Therefore, we have a mixture of recent learning and ancient knowledge, evidencing the reality of ethnopharmacology. However, popular uses of medicinal plants need scientific validation with advanced tools. Therefore, research started from the knowledge that some populations in Madagascar use decoctions of some local plants in association with low doses of chloroquine to complement chloroquine action against chronic malaria. In such a way, resistance insurgence and collateral effects are both lowered.

On the basis of the ethnobotanical work conducted by Rasoanaivo and his collaborators, 24 plants were selected and investigated for in vitro and in vivo antimalarial activity and a chloroquine-potentiating effect. In the case of validation of the activity, the determination of the active constituents followed. The results of these selections were that the alkaloids of Loganiaceae, Menispermiaceae, and Rutaceae were the most promising compounds showing significant effects, some of them potentiating the action of chloroquine.

From a phytochemical point of view, alkaloids are in pole position among natural products utilized in traditional medicine against malaria. Mono- and bis-indole alkaloids have been isolated from several plants that are traditionally used to treat malaria on different continents. The most active compounds are those that originate from plants belonging to the genera *Strychnos* (Loganiaceae) and *Alstonia* (Apocynaceae). A review covering the indole alkaloids that have high antiplasmodial activities in vitro and in vivo, and favorable selectivity indices ($SI = CC_{50}/IC_{50}$), was published by Frederich et al. (2003).

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Two important considerations attracted our attention, in view of a possibility to explore new strategies: the special endemic flora of Madagascar and the occurrence of information about a popular treatment of malaria so far never reported. Madagascar is land of endemism, consisting about 13,000 species of vascular plants, whose 80% are endemic, and even 8 families totally endemic.

Malaria is practically endemic in all Madagascar and therefore the population harbours a very rich and unique knowledge on antimalarial plants. After resurgence of malaria in the early 80's, as a consequence of *Plasmodium falciparum* resistance and due to high costs of conventional drugs, local populations back to the use of herbal remedies (Blanchard, 1901; Maggi et al., 2017). Two hundreds and thirty-nine plant species, of which about 30% are endemic to Madagascar, have been reported as having antimalarial uses in the Malagasy traditional medicine. Prof. Rasoanaivo discovered the use by some populations in Madagascar of decoctions of some local plants in association with low doses of chloroquine to complement CQ action against chronic malaria (Benelli et al., 2018). The lower use, one or two tablets of chloroquine (100–200 mg), is probably adopted to avoid the collateral effects due to a prolonged utilization of chloroquine, but such dose could be presumed inadequate to favour chloroquine resistance. Therefore, we have a mixture of recent acquirement and ancient knowledge, evidencing the actuality of ethnopharmacology. However, popular uses medicinal plants need a scientific validation with advanced tools.

Therefore, the researches started from the knowledge that some populations in Madagascar use decoctions of some local plants in association with low doses of chloroquine to complement chloroquine action against chronic malaria. In such way, resistance insurgence is lowered, as well as collateral effects. On the basis of the ethnobotanical work conducted by Rasoanaivo and his collaborators 24 plants were selected and therefore investigated for in vitro and in vivo antimalarial activity and a chloroquine-potentiating effect (Maggi et al., 2017). In case of validation of the activity, the determination of the active constituents followed. The results of this selection were that the alkaloids of Loganiaceae, Menispermiaceae and Rutaceae were the most promising compounds showing significant effects, some of them potentiating the action of chloroquine.

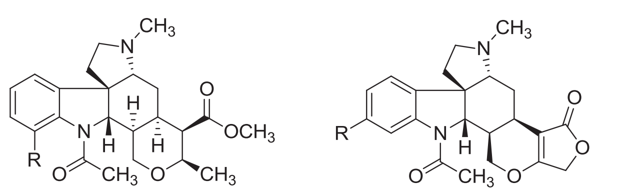
Mono- and bis-indole alkaloids are traditionally used to treat malaria in different continents (Ramanitrahasimbola et al., 2001, 2006). The most active compounds were mainly related to the genera *Strychnos* (Loganiaceae) and *Alstonia* (Apocynaceae). A review covering the indole alkaloids that have high antiplasmodial activities in vitro and in vivo, and favourable selectivity indices ($SI = CC_{50}/IC_{50}$) was published by Frederich et al. (2003).

Strychnos is a pantropic genus, with about 200 species, present in three continents: 75 in Africa, 73 in America, and 44 in Asia and Oceania (only *S. potatorum* is present in both Asia and Africa). Asiatic species are mainly small trees, whereas in the New World lianes are generally dominant. The most famous *Strychnos* species is the Asiatic *S. nux-vomica*, because of strychnine contained in the seeds with 12 other related alkaloids. Strychnine is also known and used for its bitter taste. South American species are characterized by different mono and bisindole alkaloids, important as constituents of some curare preparations of Indios Amazonia tribes (see Introduction). During the preparation of curare, the tribe curandero selects local plants and extracts the mixture by hot water. Finally, the extract is filtered on leaves and concentrated to obtain a paste, which is preserved into a container, like a calebassa or a tube, maiden by a cane, or a pottery. Active constituents in curare are bis-indole alkaloids from bark of *Strychnos* ssp. and bis-tetrahydroisoquinoline alkaloids from Menispermaceae.

The genus *Strychnos* is represented in Madagascar by 14 species, of which five are endemic to the island. Among them, *S. diplotrocha* Leeuwenberg and *S. myrtoides* Gilg & Bussse are used as antimalarial in the northeastern part of the country (Rasoanaivo et al., 2004). The phytochemical analysis allowed the separation and the structural determination of several indole alkaloids, some already known and others never reported, including mixtures of epimers, which is very unusual in the same plant (Rasoanaivo et al., 1991, 1996, 2001). The in vitro and in vivo chloroquine-potentiating effect of the crude extract of dried and powdered stem barks of *S. myrtoides* exerted chloroquine-potentiating effects on *P. falciparum* FCM29, but it was devoid of intrinsic antimalarial activity. The extract was also devoid of cytotoxic effects on HeLa and L 929 fibroblast cells. The two compounds exhibit a closely related structure but different basicity. Therefore, the latter parameter can be excluded from the factors affecting the chloroquine-potentiating effect.

These results were confirmed by other experiments, demonstrating that the crude extract of *S. myrtoides* showed higher chloroquine-enhancing activity than its major bioactive constituents. These data support the use of the plant as a phytomedicine to treat malaria, but minor components of the extract may act synergistically. Among the main isolated alkaloids, malagashanine was very interesting. Malagashanine is an unusual indole alkaloid of the *Strychnos* type. Its pentacyclic structure contains seven consecutive stereogenic centers and, most important, a transfusion between the C and D rings, against all the other similar natural alkaloids.

Therefore, malagashanine is the parent compound of a new type of indole alkaloids (Fig. 7.20) (Kong et al., 2016), named $N_bC(21)$-secocuran, isolated so far from the Malagasy *Strychnos* species, which are traditionally used as chloroquine adjuvants in the treatment of chronic malaria



Malagashanine R = H
12-Hydroxymalagashanine R = OH

Myrtoidine R = OMe
11-Demethoxymyrtoidine R = H

Fig. 7.20 Malagashanine and other related indole alkaloids from Malagasy *Strychnos*.

(Rasoanaivo et al., 1996a, 2001). Malagashanine showed only weak in vitro intrinsic antiplasmodial activity ($IC_{50} = 146.5 \pm 0.2\,\mu M$), but did display marked in vitro chloroquine-potentiating action against the FcM29 chloroquine-resistant strain of *Plasmodium falciparum*.

Another study allowed clarification of the mechanism of action of the major constituent, malagashanine, being able to prevent chloroquine efflux from the cell, and stimulates chloroquine uptake into drug-resistant *P. falciparum* strains. Malagashanine appears able to act more on plasma membrane than inside the parasite, allowing the toxicity of chloroquine against *Plasmodium*, even at sublethal doses. In the attempt to confirm the reversal of chloroquine resistance by the bark of *S. myrtoides*, a double-blind randomized controlled clinical trial of a standardized alkaloid extract titrated at 20% malagashanine took place in a government-run outpatient clinic in the town of Ankazobe (northwest central highlands of Madagascar), but the results of the treatment showed no significant efficacy, indicating a need for other confirmations. However, in conclusion, the approach, in accordance with recent tendencies on multidrug resistance control, based on mixtures of natural products and classic antimalarial drugs, with a relevant coincidence between the ethnobotanical reports and the scientific evidence, may offer interesting possible solutions for the treatment of malaria.

Many aspects about the mechanism of action of malagashanine as chloroquine adjuvant to reverse the resistance need further study. Malagashanine could increase drug accumulation by interacting with a dysregulated ion exchanger, avoiding the decrease inside the food vacuole, or acts by a mechanism related to drug binding to hematin (Perisco et al., 2017; Rafatro et al., 2000). In particular, in relation to the pH role in the blood red cell, it would be necessary to determine if malagashanine acts inside or outside the food vacuole, including the membrane periphery. The capacity of malagashanine to reverse CQ resistance may be related to the well-known properties of verapamil (Fig. 7.21) and related substances (Martin et al., 1987; Martiney

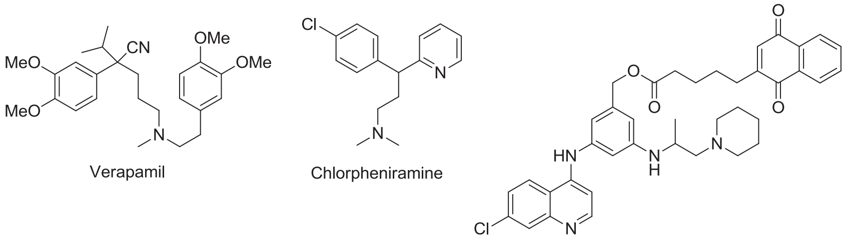


Fig. 7.21 Verapamil and other compounds studied for CQ-resistant reversal by membrane calcium channels blocking.

et al., 1995; Adovelande et al., 1998). Verapamil was the first calcium channel antagonist to be introduced into therapy in the early 1960s. It is a phenylalkylamine calcium channel blocker used in the treatment of high blood pressure, heart arrhythmias, and angina. In short-term incubations, verapamil was found to increase chloroquine accumulation in the lysosome of erythrocytes infected with both chloroquine-sensitive and -resistant organisms, but only to affect the chloroquine susceptibility of the latter. Verapamil works independently of the overall pH gradient concentrating CQ into a trophozoite's digestive vacuole. The activity is therefore related to the inhibition of membrane ion channels, interfering in the chloroquine transit within the parasite's cytoplasm. Other substances like chlorpheniramine and others are reported as candidates for CQ-resistant reversers. In any case, again the key role of natural products and ethnoparmacology information, such as for quinine (*Cinchona* sp.) and artemisinin (*Artemisia annua*), is fully confirmed.

Another attempt to explain the activity of Malagasy plants alkaloids explored the role of glutathione. L-Glutathione reduced (GSH) (Fig. 7.22) is a simple tripeptide, consisting of glutamic acid, cysteine, and glycine. It is considered one of the most powerful endogenous antioxidants, capable of preventing damage to cellular components caused by reactive forms of oxygen, radicals, and heavy metals, although its role in stress management and efficient defense against pathogens are still under study (Mangoyi et al., 2010). Besides its antioxidant defense and free radical scavenging, glutathione regenerates important antioxidants such as vitamins C and E. GSH exists in every cell of the human body, but it is also present in many other organisms, including fungi and bacteria.

There is a linkage between GSH and malaria. Some parasites are super-protected by GSH. They are endowed with powerful and host-independent mechanisms, which de novo synthesize or regenerate GSH and protect the parasites from oxidative damage and other outside attacks. GSH in particular protects the gametocytes against oxidative stress and inhibits the action of arginine, which produces NO and expels it from the food vacuole. At the trophozoite stage of *P. falciparum* in human erythrocytes, GSH takes

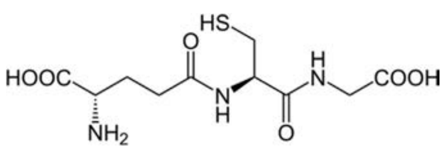


Fig. 7.22 Structure of glutathione.

part in detoxifying processes of heme, produced by hemoglobin digestion, by polymerizing some 30% of heme to insoluble hemozoin. Some authors suggest that the nonpolymerized heme, existing in the food vacuole, is subsequently degraded by GSH, increasing the role of this metabolite. Chloroquine could interact with GSH, competitively inhibiting the degradation of heme by GSH or allowing toxic heme to accumulate in membranes and damaging parasites. This argument merits some explanation. The prooxidant damage and inflammation process created by excessive heme, hemozoin, and fragments from rupture of the digestive vacuoles in blood vessels and plasma can be mitigated by glutathione. In other words, the inside of the infected erythrocyte glutathione is beneficial to the parasite; outside of the erythrocyte it reduces the negative effects of the malarial infection. High oxidative stress could actually be detrimental for the survival of young parasites (Gallo, 2009; Patzewitz and Müller, 2010).

Glutathione transferases (GSTs) are versatile enzymes involved in the intracellular detoxification of numerous substances. GSTs have been investigated in parasite protozoans, like those involved in malaria, with respect to their biochemistry and as targets in synthesis of new antiparasitic agents. *P. falciparum* possesses high quantity of these enzymes (PfGST) and their activity was found to be increased in chloroquine-resistant cells, and it has been shown to act as a ligand for parasitotoxic hemin. PfGST represents a promising target for antimalarial drug development. A PfGST isolated from *P. falciparum* has been associated with chloroquine resistance. Plant extracts have been found to act at different vulnerable metabolic sites of PfGST, disturbing GSH-dependent detoxification processes, increasing cytotoxic peroxides levels and possibly increasing the concentrations of toxic hemin in the parasites. In the case of *S. myrtoides* alkaloids, malagashanine was found to prevent chloroquine efflux from and stimulated chloroquine influx into drug resistant *P. falciparum*, suggesting that its effects are more on the plasma membrane than inside the parasite. Malagashanine (100 μM) reduced the activity of PfGST to 80%, but showed a time-dependent inactivation of PfGST, suggesting a role of malagashanine as a chemomodulator in cases of PfGST overexpression in chloroquine-resistant strains.

Future areas of research in malaria

The malaria cycle of a parasite is based on two cycles, one involving the host and the other affecting the vector. During the mosquito cycle, again there are metamorphoses and reproduction by the parasite.

In consideration of the resistance phenomenon, new transmission-blocking agents, able to interrupt malaria transmission, are required. These blocking drug components can be effective in reducing gametocyte density in the human host (gametocytocidal activity) or disrupting parasite development in the vector (sporontocidal activity), resulting in a reduced number of infective vectors and, as a consequence, decreased incidence of malaria cases. In other words, control malaria's parasites through the cure of the vectors infested by the disease.

In the sexual stages of *Plasmodium* parasites, gametocytes are critical for the transmission of the parasite to its vectors. *P. falciparum* gametocytes are also important in the disease diffusion, since being exceptionally long-lived, they cause clinically cured patients to be reservoirs of infection. The cycle of propagation of the malaria parasites starts when the female *Anopheles* feeds on blood from an infected vertebrate. Immediately, the first metamorphosis starts. By the ingestion, the mature male and female gametocytes, namely micro- and macrogametocytes, enter the mosquito host. Immediately after reaching the mosquito's midgut, the two types of gametocytes undergo dramatic metamorphoses. We must remember that such transformations are a response to environmental stimulation, like a decrease in temperature, an increase in pH, and an influence of xanthurenic acid. Within 10–20 min, the rounded macrogametes leave the erythrocytes and diffuse inside the blood, together with the flagellates microgametes. Now comes the last change. Within the next 24 h, the motile male gametes can fecundate the macrogametes, and round zygotes develop that mature to elongated motile ookinets and move to the outer midgut surface, completing early sporogonic development. These changes can be obtained by severe transformation inside the intrinsic cell organization, involving the cytoskeleton directly.

An equatorial position of chromosomes in the metaphase plate in the middle of the spindle is necessary for mitosis and symmetric cell divisions. A symmetric metaphase plate position is essential for symmetric cell divisions, explaining why it is conserved in all metazoans, plants, and many fungi. Control of this parameter is essential, since differences in cell size have been linked to cell fate and generate a class of anticancer drugs. Movements of chromosomes are in charge of microtubules, which are elements of the cytoskeleton. The cytoskeleton is a network of protein fibers forming the "infrastructure" of eukaryotic and prokaryotic cells. In eukaryotic cells, protein filaments and motor proteins form a complex mesh of protein filaments and motor proteins. The cytoskeleton aids the inside cell movement and transportation of subunits, like organelles and molecule groups, stabilizes and maintains cell shape, and gives support and order. The cytoskeleton is not a static structure but it is able to disassemble and reassemble its parts in order to enable internal and overall cell mobility. Intracellular movements include in particular manipulation of chromosomes during mitosis and meiosis from the equatorial plaque to the polar positions, in the formation of daughter cells, and also it is implicated in the immune cell response to pathogens. The cytoskeleton is composed of at least three different types of fibers: microtubules, microfilaments, and intermediate filaments. These fibers are distinguished by their size, with microtubules being the thickest and microfilaments being the thinnest. The assemblement of the proteins, tubulines a and b, makes microtubules, in form of long cave filaments. These hollow rods function primarily to help support and shape the cell and as "routes" along which organelles can move. Therefore, without the action of microtubules, the cell is unable to reproduce. The cell is blocked in a limb, with part of the mitosis already done and the final act in progress. The result is a polyploid cell, meaning a cell with double or more than the normal number of chromosomes. Because chromosomes cannot move alone, they must be dragged by the cytoskeleton. The mechanisms of action of several important antitumoral drugs derived from natural products are characterized by promotion of the assembly or disassembly of microtubules, meaning stabilization or destabilization of the tubules against depolymerization, resulting in mitotic arrest. Treated cells have defects in mitotic spindle assembly, chromosome segregation and movements, and consequently in cell division. The main problem of the utilization of these compounds in combination chemotherapy for sensitive tumor types concerns their selectivity against malignant cells. Cancer is basically a disease of uncontrolled cell division, including too-active mitosis, multiplying the cancerous mass. In most cases, these changes in activity are due to mutations in the genes that encode cell cycle regulator proteins. However, although cancer cells are a selected target, in consideration of their high level of mitosis, other tissues can be involved in the action of positive regulators of cell division. Molecular agents of plant origin are of primary importance in cancer treatment. Those acting on the cytoskeleton can be classified into two main groups: antimicrotubule agents like colchicine and the Vinca alkaloids, which induce depolymerization of microtubules, and taxol and taxotere, which induce tubulin polymerization and form extremely stable and nonfunctional microtubules (Rowinsky et al., 1990).

Neem products have been seriously explored in recent years, in several sectors, mainly in the fight against insect-borne diseases. However, it seems that so far the potentiality of neem has been only lightly touched on. NeemAzal is a marketed neem product consisting of a quantified alcoholic extract obtained from *Azadirachta indica* seeds, with a reported limonoid concentration of 57.7%, consisting of azadirachtin A 34%, azadirachtins B–K 17.7%, salanins 4%, and nimbins 2% (Dembo et al., 2015; Habluetzel et al., 2007). NeemAzal completely blocks transmission of the rodent malaria parasite *P. berghei* to *Anopheles stephensii* in vivo, when administered to gametocytemic mice at a corresponding azadirachtin A dose of 50 mg/kg. Other in vivo transmission blocking studies suggested that NA may have stronger transmission blocking activity than azadirachtin A alone, evaluating the activity of nonazadirachtin A constituents of NeemAzal. In an ex vivo assay, which exploits a major target process of azadirachtin A against *P. berghei*, microgamete formation inhibition of *Plasmodium* was used to estimate the pharmacodynamics of two varying doses of NeemAzal and azadirachtin A.

A team led by Prof. G. Chianese (University of Salerno, Italy) explored the possibility of influencing *Plasmodium* gametocytes by neem products, demonstrating the potential of blocking the reproduction stages of the parasite. NeemAzal is a marketed neem product consisting in a quantified alcoholic extract obtained from *Azadirachta indica* seeds, with a reported limonoid concentration of 57.7%, consisting in azadirachtin A 34%, azadirachtins B–K 17.7%, salanins 4%, and nimbins 2%. NeemAzal completely blocks transmission of the rodent malaria parasite *P. berghei* to *Anopheles stephensii* in vivo, when administered to gametocytemic mice at a corresponding azadirachtin A dose of 50 mg/kg. Other in vivo transmission blocking studies suggested that NA may have stronger transmission blocking activity than azadirachtin A alone, evaluating the activity of non-azadirachtin A constituents of NeemAzal.

Azadirachtins exert relevant effects on microtubules assembly and organization, interfering with the expression and/or function of adhesive proteins during the genesis of microgametocytes, through disruption of the organization of mitotic spindles and cytoskeleton formation and activity. These molecules can interfere with cytoplasmic microtubule organization and distribution, causing severe depletion of actin levels. In this action, NeemAzal proved to be more effective than azadirachtin A. In confirmation, another study showed that the product completely inhibits the growth of *P. falciparum* field isolates in *An. coluzzii* mosquitoes at a dose of 70 ppm in direct membrane feeding assays.

Microorganisms have not finished producing surprises and breaking the boundaries reported in books. Meanwhile researchers are investigating malaria parasites more and more deeply in search of their weak points, but their study is complicated by the parasite's metamorphosis, which involves not only the shape but also fundamental aspects of the metabolism (Becker and Kirk, 2004). Asexual stages of the parasite contain a single mitochondrion, whereas gametocytes can have several mitochondria. The energy production is very important. *Plasmodium falciparum*, as well as other similar Apocomplexa protozoans, possesses an intriguing non-photosynthetic plastic, discovered in the 1970s. The surprise was that apicoplastides possess their own nucleic acid. Regarding their role, they were considered by Kilejian (1991) as "a source of some substrate essential for energy production of mitochondrion." In view of their other characteristics, they could be considered a possible bridge between organisms or the ancestral point of divergence from green algae and protozoans. In conclusion, apicoplastides could be part of the endosymbiosis pathway, wherein degenerated chloroplasts were useful to increase a mitochondrial efficiency still in evolution. Thus, endosymbiosis started with the inclusion of the two main bacterial forms, the hetero- and the autotrophic one, but later the ancestral (green or red) primordial alga degenerated the chloroplast in favor of a clear evolution toward the heterotrophic metabolism (Fig. 7.23).

Usually the shift to the eukaryotic cell is considered a consequence of environmental factors, such as the increase of oxygen in the oxidative atmosphere; however, it is possible that in some cases interactions between organisms could also have played an important role.

The study on apicoplastides allowed researchers to evidence similarities (Keeling, 2008; Kilejian, 1975; Köhler et al., 1997) between different arthropod-borne diseases, such as avian malaria, eimoriosis, and toxoplasmosis, confirming once more the occurrence of common survival strategies in different organisms. Other differences concern the enzymes network and the membrane transport mechanisms. The new knowledge about parasite-specific organelles could be of fundamental importance to the development of future antimalarial drugs, increasing efficiency and decreasing side effects, like resistance.

Another important research front full of possibilities is focused on the membrane mechanism of CQ's extrusion by permeability pathways induced by the parasite in the host red blood cells (Saliba et al., 1998). This is related to the CQ of interfering with the detoxification of toxic heme monomers.

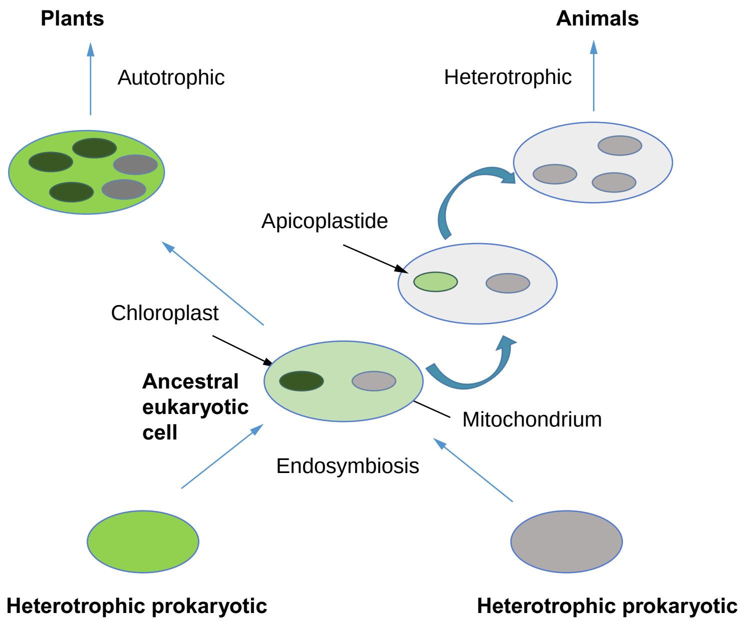


Fig. 7.23 The possible role of apicoplastide in the endosymbiosisi.

The studies showed that 12–16 h after the invasion by the parasite, the so-called new permeability pathways act on the interchanges, i.e., the entry of nutrients, as well as mediating the efflux of metabolic wastes. Several groups advanced the hypothesis of a number of channel types, activated by particular stress or stimuli (Ginsburg and Stein, 2004; Kirk et al., 1994; Duranton et al., 2004; Staines et al., 2004; Thomas and Lew, 2004).

All these references testify to and confirm the presence of a wide range of studies in search of an answer to the challenge of resistance. The front is still too large and undetermined, but every year the knowledge of host cell reaction is increasing and there is a high probability that the problem will be solved in the coming years.

Conclusions

During the development of the arguments contained in this book, it was necessary link the insect-borne diseases argument to several collateral items. The idea in particular was concentrated on a possible utilization of this particular topic as an epiphany, meaning an enlightening subject, which allows a revision of the problem from a new perspective. The interpretation of a new and key piece of information can allow the process of significant thought about a problem, until, in accordance with the original significance of the term in ancient Greek, the ἐπιφάνεια (*epiphanea*) appears like a manifestation, with a striking appearance.

This book started with considerations about gaps and books. Let us now return to these two points.

The lesson from Carson's book about the fundamental role of beneficial insects in the survival of mankind, has arguably not been understood. Throughout all warm terrestrial ecosystems, insects are a dominant component and they are part of the lives of any organism. The insect-plant relationship is a fundamental biotic interaction, and plants account for a large part of the planet's biomass, many times the biomass of all animals together (New, 2002; Jankielson, 2018; Dunn, 2005). The animal biodiversity is dominated by that of insects. They are a beautiful example of variability, in terms of both number of species (more than 1 million) and abundance (more than half of all living organisms), although at most only about 7%–10% of insects are scientifically described. This diversity, consisting of large numbers of individuals and great intra- and interspecific variety, is a consequence of the enormous functional significance of insects in habitats. Primitive insects appeared very early in the Silurian period, when plants and animals finally emerged from the sea and colonized dry land, and over the last 400 million years the number of insect families has been rising. They were able to colonize any part of the territory, including the sky. Today, the number of reported insect families is about 600 and they have survived various negative major impacts, including the mass extinction event at the end of the Cretaceous period. A review analysis, published in the journal *Biological Conservation* by Francisco Sánchez-Bayo, at the University of Sydney, Australia, and Kris Wyckhuys, at the China Academy of Agricultural Sciences, Beijing China, attests a current insect collapse. The decline's hypothesis is based on a study of 73 recent selected studies. The causes and significant factors include intensive agriculture, the heavy use of pesticides, urbanization, and climate change. The loss of insect population is calculated in an annual 2.5% rate over the last 20–25 years, and the future tendency is evaluated to 25% in the next 10 years and increasing continuously until only half left in 100 years. This scenario is already underway. In Puerto Rico, a recent study revealed a 98% fall in ground insects over 35 years. The catastrophic cascade effects on the planet's ecosystems include ants, aphids, shield bugs, and crickets, which are the food for many birds, reptiles, amphibians, and fish that eat insects. There are many indicators supporting the scenario (Sánchez-Bayo and

Wyckhuys, 2019; Diamond, 1989). In England, between 2000 and 2009, the number of widespread butterfly species fell by 58% on farmed land, suffering the biggest recorded insect falls overall—though that is probably a result of this area being more intensely studied than most places. A particular alert concerning bees being seriously affected has also been raised in Europe and the USA; for example, only half of the bumblebee species found in Oklahoma in the USA in 1949 were present in 2013 (Alburaki et al., 2015, 2018; Aizen, 2009). The number of honeybee colonies in the USA was 6 million in 1947, but 3.5 million have since been lost. In 2013, according to EU data, there were around 630,000 beekeepers and 16 million hives in the EU, producing 234,000 tons of honey per year, but the same source tells us that many insect pollinator populations are now in clear decline. There is similar news from Brazil, with half a million bees dead. On one side, this is considered the effect of the use of some pesticides, toxic to bees. On the other side, it is a classic example of rapid and intense environmental change to improve agricultural intensification and pasture, with the systematic elimination of all trees and shrubs that normally surround the fields, so there are plain, bare fields that are treated with synthetic fertilizers and pesticides.

Dr. Sanchez-Bayo said: "We are not alarmists, we are realists. We are experiencing the sixth mass extinction on Earth. If we destroy the basis of the ecosystem, which are the insects, then we destroy all the other animals that rely on them for a food source." He added, "It will collapse altogether and that's why we think it's not dramatic, it's a reality."

The situation comprehends micro- and macroepisodes, like the continuous devastation of equatorial tropical forests, in particular the Amazonia territory. The sequence is clear and well-known, and it always works: first, the fire destroys the vegetation, in particular the woody plants; second, the soil is cleaned, otherwise the plants could replace the habitat rapidly; and third, the territory is declared totally compromised and ready for further utilizations. However, as observed by Samways in *Biodiversity and Conservation* (1993, and later confirmed by this author in a series of further papers) in a paper titled "Insects in Biodiversity Conservation: Some Perspectives and Directives," the main concerns are the "Lack of human appreciation of importance, coupled with the general disregard and dislike of insects, is an enormous perception impediment to their conservation. This impediment coupled with the taxonomic impediment must be overcome for realistic biodiversity conservation management. As it is not possible to know all the species relative to the rate at which they are becoming extinct, it is essential to conserve as many biotopes and landscapes as possible." There is a sentiment of urgency for measures "essential to preserve species dynamo areas as an insurance for future biodiversity," such that "preserved areas must also be linked by movement and gene-flow corridors as much as possible." The last point of view is crucial. Preservation must be considered not only as an opportunity to maintain the presence of species in selected habitats against their disappearance, but it must be considered changes as opportunities to perform a positive future. In this regard, entomologists are asked to contribute in control of vectors affecting humans, crops, and livestock, but also to take an active part in the consideration due to the beneficial species.

The central task is the possibility to predict accurately the environmental effects of any intervention. Once the inherent risks connected with traditional control methods have been considered, the consequences of new introductions must be carefully predicted, including any synergist effect. The rate of insect species extinction is estimated as being eight times faster than those of mammals, birds, and reptiles (Barnosky et al., 2011; Dirzo et al., 2014).

Another important current gap concerns scientific information. Most ordinary people do not have access to data obtained by the scientific community, as well as opinions and models produced by experts and scientists. Information, when available, is usually distorted and adapted to the dominant axioms by a plethora of generalist supposed experts. The proposed idea is that these kinds of people are able to know and comment on everything. The distortion, sometimes voluntarily pursued and often a consequence of general confusion, generates progressive modification of the starting points and even the concealment of important facts. The recent phenomenon of fake news is clearly generated from the same situation. Although most research information is now easily accessible and can be obtained directly from the internet, its utilization remains restricted to dedicated people. In contrast, some scientific information is amplified far away from its real impact. How many times did you read about the discovery of a definitive cure to cancer? Or about the already obtained solution to any physiological problem using staminal cells? In our era of globalized knowledge, news are obtained and fluxed indirectly, without few possibility of checking the origin and the reliability. It is necessary to consider that more than 46% of the human population, consisting of 3.5 billion people, are connected via the internet, and 2.5 billion utilize social networks regularly. These numbers are likely to increase 10% every year. All these people have access to information only through selected channels and although they are in a condition to verify it, science and general information are on different and distant levels. The main problem is that the information is reduced to a few soundbites, and there is no place for elaboration or proposals of other possible interpretations or points of view. This is not a recent case, produced by digitalization of communication.

Beside the sources, the problem of the quality of scientific information was fully evidenced more than 30 years ago, in the "Public Understanding of Science." This is the title of a report requested in 1985 by the Royal Society and prepared by a group of experts, whose leader was the geneticist Sir Walter Fred Bodmer. The report evidenced the general lack of knowledge about scientific themes. On one side, most of the population, accounting for two-thirds of Europeans, was confident about science and technologies, considering that scientists were able to solve human problems and make human life "easier, healthier and comfortable." On the other side, the sequence "more communication = more knowledge = more social adherence to scientific arguments" appears largely inadequate. The dominant problem about scientific communication is that ordinary people need an alphabetization to understand and meet the complexity of the scientific items. The conversion of the original scientific information is usually distorted and changed, at best "adapted," but more often polluted by political, social, and cultural interests. The result is a reductive metamorphosis, in the best case, or complete revision to be adapted and useful to already-made opinions. Among the various examples of this operation we find never-ending debates, such as those concerning OGM, vaccines, or the consequences of climate changes, without considering abnormal and artificially created themes, such as the contraposition between vegans and meat-eaters. The manipulation is based on a presumed "democratic" interpretation of scientific data. No vote is necessary to assure the consistency of a scientific law based on adequate experimentation, but the aim is that reliability must be obtained by public consensus and even agreement. Independence has always been a necessary character of science, but manipulation was never pursued. History tells us that any political or social manipulation of science led to disaster. In contrast, priorities, when based on correct scientific information, as well as consequent implications and decisions, must be subject to the most ample democracy.

At the end of this little journey through macro-, micro-, and nanoworlds, it is undeniable how long the road still is to understand and discover the mysteries of insect-borne diseases. In the meantime, we await the next surprises. The COVID-19 pandemy dramatically evidenced all the current limits of science and technology to face this kind of challenges. The virus was faster and clever. Predictively and prevention were insufficient. Despite the potentiality, the debacle and medicine was evident and the consequent economic and social damages were enormous. Microorganisms will continue to play their role inside the habitats and next time their target could be the industrialized sources of our food. However, it is clear that is society will continue to ignore the alerts of researchers and scientists, the next pandemy will be the worst one.

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