16 Guidelines for the Nonclinical Evaluation of the Efficacy of Traditional Antimalarials

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16.1 INTRODUCTION

For the foreseeable future, chemotherapy and impregnated bed nets will remain the two most useful tools for the control of the deadly disease malaria, which kills 2 million people each year. Paradoxically, only approximately 10 antimalarial drugs are available on the market for the prevention or the treatment of malaria, and the development of new ones is costly and time-consuming. The use of chemotherapy for controlling the pathogenic organism is further restricted by the development of drug resistance. As there is no longer a single drug that can prevent or cure all cases of malaria, researchers have reconsidered the entire therapeutic approach for the control of this old and most devastating tropical disease. A more flexible attitude should be adopted to this end. Whereas the urgent need for the discovery or design of new antimalarial drugs with different mechanisms of action is recognised, plant-based antimalarials form the basis of medicines used by the majority of people in most regions afflicted with malaria. Many have been shown in experimental studies to have antiplasmodial effects, and as such, they may offer viable alternatives to prescription drugs in the treatment of this life-threatening disease.

Before a traditional antimalarial plant can be used in primary health care, however, it is essential to adequately assess its efficacy. Indeed, insufficient evidence of the efficacy of an herbal product is not acceptable, particularly when that product may entail serious health risks. Evaluating the efficacy of antimalarial plants may be viewed at two levels. The first is to demonstrate that, in the form in which they are used in traditional medicine to treat malaria, they have beneficial effects. The second, assuming that chemically defined constituents are responsible for the observed activity, is to isolate these constituents for further investigation. Based on our own experience and our exchange of views with colleagues involved in a malaria research program, we will propose in this chapter guidelines for the preparation of extracts of plants, bioassay with crude extracts, and bioassay-guided fractionation procedures.

16.2 AN INTEGRATED APPROACH TO ANTIMALARIAL PLANTS

When dealing with traditional medicine, it is important to bear in mind that healers basically treat the symptoms of a disease, especially those that are apparent to them. As malaria can produce a wide variety of symptoms, over 1200 plant species are used to treat this disease (see Chapter 11). Medicinal plants considered effective in the treatment of malaria are therefore those observed by healers to alleviate or prevent one or more recognised symptoms of malaria. As malaria can occur concurrently with other infectious diseases, accurate diagnosis can be difficult to achieve, and this makes malaria symptoms somewhat complex. Ethnomedical beliefs of populations also play a role in the choice of plants for the treatment of malaria. Based on these considerations, antimalarial plants can be roughly divided into three categories:

1. Plants with a direct effect on the parasite, either at the erythrocytic stage (antiplasmodial drugs) or at the hepatic stage (preventive drugs)
2. Plants with effects on host–parasite relationships (immunostimulants, antipyretics, etc.)
3. Plants with no clear effects on malaria, but with probable psychosomatic action, the use of which originates from ethnomedical beliefs

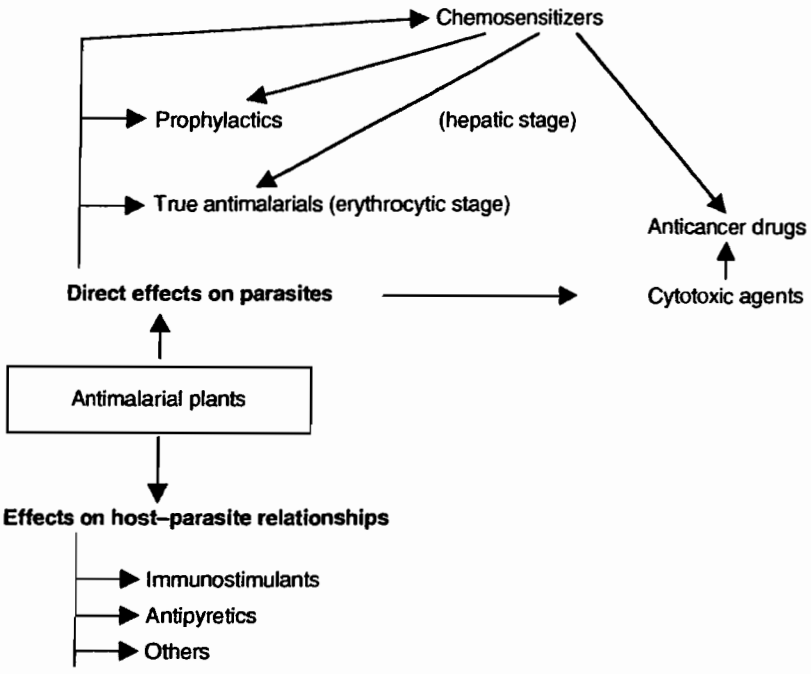

FIGURE 16.1 Components of the integrated approach based on known or probable biological activities of antimalarial plants.

This has led us to propose an integrated approach for investigating antimalarial plants (Rasoanaivo, 2002). The computerised compilation of all ethnomedical uses of Madagascan medicinal plants for the period 1500–2002 (Rasoanaivo, 2000) provides a good illustration of this approach. For example, medicinal plants used to treat malaria in one region are reported to be used as tonics in another, and even claimed to be toxic in yet other parts of the island. Similar variations in the use of medicinal plants may also be encountered in other countries. This integrated approach is summarised in Figure 16.1.

Which of these therapeutic lines should be prioritised when evaluating the efficacy of antimalarial plants? In our opinion, effort must of necessity be selective, focusing on one or two relevant biological activities. Progress in our understanding of the biology and biochemistry of malaria parasites during the last two decades clearly shows that the erythrocytic stage of *Plasmodium* is by far the most important target in malaria chemotherapy (Olliaro and Goldberg, 1995). We therefore suggest that antimalarial plants should first be evaluated for their ability to kill the parasite in the blood stage. Another important target is the hepatic stage of *Plasmodium*, in which there is a serious lack of appropriate drugs.

For efficacy evaluation, three key points must be considered: the extraction procedure, the choice of appropriate malaria-oriented bioassays, and the fractionation procedure.

16.3 PREPARATION OF EXTRACTS FOR ANTIMALARIAL SCREENING

A wide range of methods for extraction and preliminary fractionation are described in the literature dealing with plant chemistry. They are generally designed for a specific purpose such as pure phytochemical characterisation, biological screening, bioassay-guided fractionation, and selective extraction of one or more compounds. In demonstrating biological activities of medicinal plants, the choice of appropriate extraction and fractionation procedures is of paramount importance. We

think that some projects have failed to detect useful compounds in medicinal plants in part because of shortcomings in the procedures employed for preparing the plant material for analysis.

16.3.1 Plant Material

First of all, the botanical identity of the antimalarial plants collected must be authenticated by a taxonomist. So many mistakes have occurred in the past in this respect that proper identification of the plant material should be regarded as an essential prerequisite. It is also common practice to keep voucher specimens of plants examined at the institute where the work is carried out. Although generally omitted, the details of plant harvesting conditions, such as time of day, season, and stage of plant development, should be recorded in order to work out the optimum conditions for collecting the plant material.

Plant parts collected should be those used in traditional preparations. Ideally, fresh plants should be used to prevent enzymatic degradation or hydrolysis from occurring. Alternatively, plants may be dried before extraction, especially if they are not used fresh. If this has to be done, it is essential that the drying operation be carried out under controlled conditions so as to minimise postharvest chemical changes. To this end, plants should be dried as quickly as possible without using high temperature or sunlight, and preferably air-dried. Once thoroughly dried, plant material can be stored for long periods of time before analysis.

The plant material collected must be free from contamination with other plants. Mosses often grow in close association with higher plants, and it is essential to remove them. In the case of higher plants, mixtures of plants may sometimes be gathered in error. Additionally, two closely similar plants growing side by side in the field may be incorrectly assumed to be the same, or a plant may be collected without realising that it has a parasite intertwined with it. It is important to collect plants that are not affected by viral, bacterial, or fungal infections. Not only may compounds derived from microbial synthesis be detected in such plants, but also infection may seriously alter plant metabolism and unexpected products could be formed, possibly in large amounts.

16.3.2 Extraction Procedure

16.3.2.1 Ethnopreparation-Based Extraction

Traditional healers have their own methods for preparing antimalarial recipes, some of which have proved to be efficient and safe for use in humans over the thousands of years they have been in use. However, the mode of preparation of the formulation currently used by healers is commonly ignored when evaluating the efficacy of medicinal plants. Traditional antimalarial remedies should be prepared exactly as they are used in traditional medicine, preferably in close collaboration with healers, and used as such while evaluating them using *in vitro* and *in vivo* experimental models.

We wish to report here an interesting case that deserves attention. Ten years ago, we investigated a medicinal plant of Madagascar, *Strychnopsis thouarsii* baill. (Menispermaceae), which has been traditionally used in decoction form as an antimalarial for a long time, and as a chloroquine enhancer more recently. At that time, we were focusing on drugs that reverse chloroquine resistance in malaria. We were able to isolate alkaloids with chloroquine-potentiating effects (Ratsimamanga-Urverg et al., 1992). As these alkaloids did not show any promising results for drug development, we discontinued this work. At the inaugural meeting of Research Initiative on Traditional Antimalarial Methods (RITAM) in Moshi in 1999, we became aware of the availability of *in vitro* and *in vivo* tests at the hepatic stage of the malaria parasite. Our colleagues in Paris have since tested the decoction of the plant prepared in the traditional way and have found that this decoction killed hepatic schizonts (see Chapter 17). Bioassay-guided fractionation

has shown that the active constituents would be located in the polar fraction. These results, as yet unpublished, clearly show that we would have missed interesting compound(s) if we restricted our work to easily isolated alkaloids without taking into consideration the traditional preparation as used by the local population.

If the extracts display significant antimalarial activity either in the preerythrocytic stage or in the erythrocytic stage of the malaria parasite, isolation of the active principles is considered a logical next step. On the other hand, lack of antimalarial activity in these two experimental models does not necessarily imply that the traditional remedies concerned must be discarded. A strategy for handling this possibility is proposed in the last paragraph of this chapter. It is suggested that observational clinical studies, discussed in detail elsewhere in this book, should be carried out instead, to assess the reported beneficial effects of the traditional preparations.

16.3.2.2 Solvent Extraction

In some cases, the plants used as antimalarials are known from the literature, but the exact recipe for preparing the traditional remedy is not clearly described. Scientists generally avoid using water extraction, which is the method used by healers in most cases, because of the complexity and difficulty involved in developing a suitable workup procedure with aqueous extracts. Organic solvent extractions are therefore used as a good alternative in evaluating the antimalarial activities of plants. To this end, alcohol or aqueous alcohol, in any case, is a good all-purpose solvent for preliminary extraction in a screening program. Particularly, methyl or ethyl alcohol has the ability to extract a broad spectrum of chemical substances. It has been used in screening programs searching for antimalarial compounds from plants (Leaman et al., 1995; Muñoz et al., 2000; Simonsen et al., 2001). In this single-solvent extraction procedure, the plant material is subjected to extraction exhaustively, by repeated maceration with alcohol or aqueous alcohol at room temperature. The alcohol fraction in the combined extracts is evaporated off under reduced pressure at a temperature not exceeding 45°C, and the residual water extract is freeze-dried or evaporated to dryness by azeotropic methods by repeatedly adding 95% ethanol to the residual water until this water is completely removed. As a general rule in our laboratory, approximately 25 g of dried plant material is used for extraction in the primary screening. Extraction with alcohol in a Soxhlet apparatus has been reported for various parts of an antimalarial plant (Sharma and Sharma, 1999), but in our own work we avoid the use of this technique because extracts are continuously boiled with the solvent for several hours, which may alter labile constituents.

Successive extractions with solvents in increasing order of polarity are also a useful practice followed in several laboratories (Gessler et al., 1994). In this procedure, plant material is defatted with petroleum ether, cyclohexane, or heptane, the use of hexane being avoided because of its toxicity and flammability. The residual powdered plant is then extracted, preferably with ethyl acetate because of its lower toxicity compared to chlorinated hydrocarbon solvents, or alternatively with dichloromethane or chloroform. Thereafter, the residue is extracted with methanol or ethanol, and finally with water. This procedure is based on the old Roman principle of solubility: *similia similibus solvuntur* (the similar dissolves the similar). Scientifically speaking, nonpolar solvents dissolve selectively nonpolar compounds, and polar solvents dissolve preferably polar compounds. A reasonable alternative is to shorten the procedure by using only one nonpolar solvent (ethyl acetate) and one polar solvent (methanol or water).

When using antimalarial screening tests involving inhibition of enzymes, all tannins and polyphenols should be removed from the extracts in order to avoid false positives due to interference with the enzymes. This is achieved preferably by passage through a polyamide chromatography column, or alternatively by precipitation with polyvinylpyrrolidone.

16.4 BIOASSAY OF PLANT EXTRACTS FOR ANTIMALARIAL ACTIVITY

In malaria chemotherapy, most research has been aimed at searching for drugs that could kill the malaria parasite in the erythrocytic stage of its development. Methods used for evaluating the efficacy of drugs, i.e., basic assessment of antiplasmodial activity or mechanism-based assays, have therefore been designed to meet this purpose. There are basic tools used in a primary screening to assess the ability of herbal antimalarials to kill the malaria parasite in the asexual stage. Regarding the hepatic stage, tests are also available for use in screening programs (see Chapter 17). The following paragraphs will be dedicated to experimental models for detecting antiplasmodial activity of plant extracts in the blood stage of the malaria parasite.

16.4.1 Experimental Models for Detecting Antiplasmodial Activity of Plant Extracts in the Erythrocytic Stage of Malaria Parasites

16.4.1.1 *In Vitro* Antiplasmodial Tests

The radioactive microdilution technique originally developed by Desjardins et al. (1979) and modified by Le Bras and Deloron (1983) and O'Neill et al. (1985), and later on by several scientists around the world to suit specific screening purposes, has proved to be very useful for the preliminary evaluation of *in vitro* antiplasmodial activity of plant extracts. It is based on the inhibition of tritiated hypoxanthine uptake by *Plasmodium falciparum* cultured in human blood. It is accurate, rapid, reproducible, easily automated, and only requires small amounts of extracts, which makes it well suited for bioassay-guided fractionation. Although it has been difficult to adapt this technique for high-throughput screening due to the limiting factor of culturing the parasite, one of its advantages is the possibility of discovering bioactive molecules with unexpected or novel mechanisms of action. The results are expressed as percentage inhibition with respect to controls for one single dose, generally 10 μg/ml, or as median inhibitory concentration (IC_{50}) obtained by linear regression methods (Huber and Koella, 1993). Its disadvantages include the need to use tritiated hypoxanthine (which is hazardous and expensive) and a liquid scintillation counter. Alternatively, viable parasites in each well can be stained with Giemsa or Diff-Quick® (a rapid staining kit) reagents and examined under the microscope. The parasites on each blood film are counted using a high-power microscope lens with oil immersion, and the percentage of growth inhibition with respect to the control is determined by a simple arithmetic calculation.

Colorimetric methods have also been developed and applied successfully to the assessment of *P. falciparum* drug susceptibility. These include the parasite lactate dehydrogenase (LDH) assay (Makler et al., 1993) and the microculture tetrazolium assay (Delhaes et al., 1999). These two enzyme assays are nonradioactive, rapid, reliable, and inexpensive to perform, suggesting their suitability for application in the screening of antimalarial drugs. However, to the best of our knowledge, there has hitherto been no report on the use of these two methods for the screening of plant extracts for antiplasmodial activity. At this point, further investigation is needed before they can be applied as routine methods.

In vitro tests are also useful for the detection of drug interaction, i.e., antagonism, synergism, and simple additive effects, by the isobologram method (Rasoanaivo et al., 1994). However, they have some limitations because they are not necessarily predictive of *in vivo* activity.

16.4.1.2 Mechanism-Based Assays

The progress in our understanding of the biology and biochemistry of malaria parasites during the last two decades has led to the identification of drug targets that are both parasite specific and essential for parasite growth and survival. There are several recent reports on chemotherapeutic targets for antimalarial drug discovery and development (Olliaro and Yuthavong, 1999; Jomaa et al.,

1999; Macreadie et al., 2000). Particularly, the heme polymerisation process has attracted much attention as a valid drug target (Monti et al., 1999; Kurosawa et al., 2000). However, one limiting factor is the use of the expensive radiolabeled ^{14}C-hematin in the experimental procedure. Recently, it has been shown that the detoxification mechanism of hematin in malaria parasite involves not a polymerisation, as was previously believed, but a dimerisation of hematin, which is called biocrystallisation (Hempelmann and Egan, 2002). This has led to the development of simple, nonradiolabeled techniques to assess extracts/compounds for their ability to inhibit the biocrystallisation process (Deharo et al., 2002; Sahal et al., 2003; Steele et al., 2002).

16.4.1.3 Cytotoxicity Tests

Cytotoxicity tests are not designed to screen extracts for antimalarial activity. The malaria parasite, like all living organisms, undergoes cell division from merozoites to schizonts. Extracts and compounds that inhibit cell division may also kill the parasite. Cytotoxic natural products may therefore give false positive results for antiplasmodial activity in screening programs using either the *in vitro* radioactive or the colorimetric methods. Any extract that inhibits the growth of *P. falciparum in vitro* should therefore be tested systematically for cytotoxicity. There are several techniques currently available (Husoy et al., 1993). To estimate the potential of a given extract to inhibit parasite growth without host toxicity, the selectivity index (SI) was introduced and defined as the ratio of IC_{50} in cytotoxicity to the IC_{50} in *P. falciparum*. The higher the SI, the higher is the selective antiplasmodial activity of a given extract or compound.

16.4.1.4 *In Vivo* Antiplasmodial Activity

The reference test for the blood schizontocidal activity of plant extracts is the 4-day suppressive test of Peters et al. (1975) using the rodent malaria model (see also Peters and Robinson, 1999, for more details). It evaluates the reduction in parasitaemia of mice infected by rodent malaria parasite following administration of a daily dose of plant extract. It is also useful to evaluate drug interactions. Results are expressed as percent of parasitaemia inhibition with respect to untreated controls. One hundred percent parasite inhibition is called parasite clearance.

Whenever possible, each *in vitro* or *in vivo* test involving the erythrocytic stages of *Plasmodium* malaria should include a positive control with a *Cinchona* sp. (*C. ledgeriana*, *C. succirubra*, *C. calisaya*). These plants offer the possibility of calculating the activity index (AI) defined as IC_{50} of a plant extract/IC_{50} of *Cinchona* sp. extract. If AI = 1, the plant is as active as the reference; if AI < 1, the plant is more active than the reference; if AI > 1, the plant is less active than the reference. This index would make results comparable from different laboratories.

16.4.2 Ranking the Efficacy Results of Antimalarial Extracts

Assuming that antimalarial plants are effective in treating the disease, those acting on the erythrocytic stage of the malaria parasite are expected to have significant *in vitro* antiplasmodial activity. In a previous paper, we ranked the level of efficacy of extracts according to their IC_{50} values (Rasoanaivo et al., 2002). The proposed thresholds for *in vitro* antiplasmodial activity are summarised with some modifications in Table 16.1.

For single-dose testing, extracts that inhibit 80% or more of the parasite growth with a concentration of 10 µg/ml are considered worthy of further bioassay-guided fractionation.

Regarding *in vivo* activity in rodent malaria models, parasite clearance without recrudescence for a period of 60 days would be the ideal criterion. The suggested thresholds for *in vivo* antiplasmodial activity for a dose level of 250 mg/kg/day are listed in Table 16.2.

With respect to the selectivity index, SI > 300 should be attained on the basis of mean values reported in relevant papers (O'Neill et al., 1985; Schrével et al., 1994; Angerhofer et al., 1999).

TABLE 16.1
Proposed Thresholds for *In Vitro* Antiplasmodial Activity of Antimalarial Extracts

IC_{50} (µg/ml)	Level of Activity
<0.1	Very good
0.1–1.0	Good
	This is the concentration range that is generally considered active in screening programs for antimalarial activity, warranting bioassay-guided fractionation
1.1–10	Good to moderate
	This range may reasonably be considered for bioassay-guided fractionation
11–25	Weak
26–50	Very weak
>100	Inactive

TABLE 16.2
Proposed Thresholds for *In Vivo* Activity of Antimalarial Extracts at the Dose of 250 mg/kg/day

% Inhibition	Level of Activity
100–90	Very good to good activity
90–50	Good to moderate
50–10	Moderate to weak
0	Inactive

Ideally, extracts effective at the blood stage of the malaria parasite should have strong *in vitro* and *in vivo* antimalarial activities and should be devoid of cytotoxicity at concentrations of up to 100 µg/ml. Priority should be given to the safety evaluation of such extracts with the objective of conducting clinical trials of the traditional medicine or a derived phytomedicine. Extracts that meet these criteria also deserve further investigation aimed at isolating the bioactive compound(s), which may serve as candidate(s) for drug development in the Western pharmaceutical context.

16.5 BIOASSAY-GUIDED FRACTIONATION

It has been widely recognised in screening programs involving plant extracts, and our experience in screening for antimalarial activity confirms this (Rasoanaivo et al., 2002), that the percentage of plants with confirmed activity could be significantly increased if the plants are subjected to preliminary fractionation before screening (Statz and Coon, 1976). It is therefore advisable that extracts with moderate activity be subjected to bioassay-guided fractionation. If reasonably good antimalarial activity is not obtained at this stage, further work is discontinued since activity rarely improves significantly as additional fractionation progresses. During the phytochemical study, continuous monitoring for antimalarial activity is conducted with all the fractions obtained, avoiding exhaustive isolation and structure elucidation for those compounds not related to the antimalarial activity detected initially.

Fractionation should be initiated using simple methods, which entail minimum risk of destroying or altering active principles, thus leading to useless artifacts. As far as possible, no chemical reaction of any kind should be utilised during the fractionation procedure. The one exception is the conventional acid–base treatment when the active constituents are alkaloids that are presumably unaffected by this treatment.

When the single-solvent extraction procedure is used, there are several alternative methods of fractionation to choose from depending on the equipment available in the laboratory. Four different methods with which we have some experience in our screening programs are described in the next paragraphs.

16.5.1 Solvent Partition

Solvent partition is the simplest and least expensive method for a preliminary fractionation. Generally performed in a separating funnel, this method is based on the differential solubility of compounds between two immiscible solvent phases. It is used as early as possible in the processing of plant material in order to remove the maximum quantity of inactive constituents. An important feature of this method is its flexibility/adaptability, offering a good range of options for a preliminary fractionation. Modification or adaptation can be done gradually, at any time during the screening program, with the aim of developing a procedure that would give the highest percentage of active compounds for a given sample of antimalarial plants. Figure 16.2 shows a general procedure we presently use in our institute for extraction and solvent partition in screening programs.

Initially we used chloroform as a solvent of medium polarity (Rasoanaivo et al., 1999), but the reported toxicity and the difficulty in obtaining this hazardous chemical led us to discontinue its use in favor of ethyl acetate (Rasoanaivo et al., 2002). Our procedure is flexible, and in most cases ethanol extracts and ethyl acetate fractions are systematically tested. The other fractions are considered when ambiguity occurs in the test results.

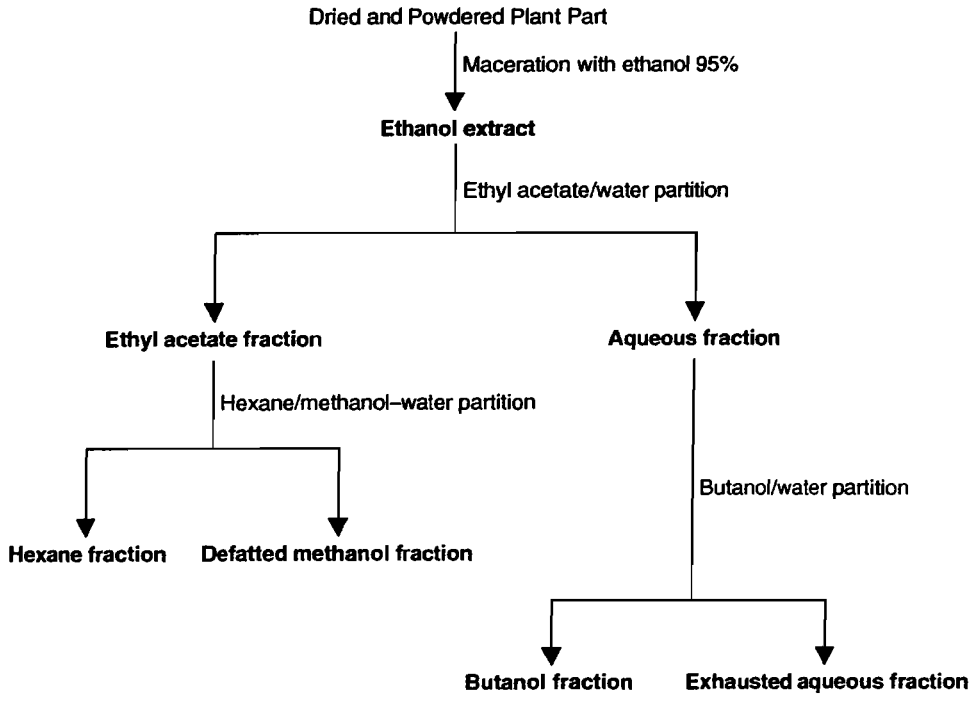

FIGURE 16.2 A general procedure used by Institut Malgache de Recherches Appliquées (IMRA) for extracting plant material and fractionating into different solubility classes.

Successive solid–liquid extractions using solvents in increasing order of polarity represent an acceptable alternative to liquid–liquid partition.

16.5.2 Countercurrent Distribution

Although some of the inactive constituents are probably removed by solvent partition, fractions are still a mixture of several compounds. It is known that the biological activity is not always due to the main constituents but sometimes to minor ones, or even to the synergism of active and inactive constituents. One of the best methods for further fractionation of active extracts employs the countercurrent distribution (CCD) technique performed using an automated Craig apparatus. The principle of fractionation involves the partition of each of the substances in a mixture between two immiscible solvent phases. Basically, CCD fractionation is similar to solvent partition in a separating funnel, but the operation is automatically repeated several times in terms of transfers in the Craig apparatus. The choice of a suitable two-phase solvent system is crucial for a successful fractionation with CCD (Galeffi, 1980). After a judicious mixture of organic solvents and water is made, 100 to 200 transfers are run and fractions are gathered according to similarity pattern in thin-layer chromatography (TLC) and systematically tested. Further CCD fractionation can be performed on the active fraction(s) to isolate the active constituent(s). This technique presents several advantages over the solid–liquid fractionation methods: (1) no irreversible adsorption, (2) total recovery of introduced extract, (3) possibility of isolating very minor constituents with close polarity, and (4) minimum risk of sample degradation. Our Italian colleagues at the Istituto Superiore di Sanità in Rome have used this technique extensively for the bioassay-directed fractionation/separation of plant extracts. We have benefited from their expertise in the isolation of the minor cytotoxic constituents of *Kalanchoe tomentosa* (Rasoanaivo et al., 1993) and *Millettia pervilleana* (Galeffi et al., 1997) and in other work.

16.5.3 Solid–Liquid Column Chromatography

Silica gel column chromatography is a good method for fractionating active extracts if the compounds have a low to medium polarity. We have preferentially used this technique in most of our bioassay-guided fractionation. To this end, a coarse fractionation is first done using a system of solvents with increasing polarity, and the collected fractions are examined by TLC. Fractions with similar TLC patterns are combined and monitored by antimalarial tests. Active semipurified fraction(s) are further subjected to a more refined column chromatography.

In some cases, a combination of two or several analytical techniques is necessary to adequately locate and isolate the antimalarial substances. Samuelson and co-workers (1985, 1987) described a fractionation procedure for crude aqueous extracts using a combination of solvent partition, ion exchangers, and gel filtration, all fractions being subjected to bioassay. This method gives useful information about the physical properties of the active constituents, which might enable the development of suitable isolation methods.

16.5.4 Preparative HPLC Fractionation

Preparative high-performance liquid chromatography (HPLC) could be used on a routine basis to fractionate extracts before subjecting them to bioassay (Sevenet et al., personal communication). However, the equipment required and the cost of columns that must be replaced every 40 passages would make this technique beyond the reach of many laboratories.

16.6 PROBLEMS ENCOUNTERED IN THE ANTIMALARIAL TESTING OF PLANT EXTRACTS

Plant extracts are generally mixtures of several compounds. Their composition as well as the concentration of individual components may vary depending on ecological conditions. Most pharmacological screening problems arise from this.

16.6.1 Failure to Isolate Active Constituent(s) from an Active Extract

In a bioassay-guided fractionation, failure to isolate active constituents from an active extract is often a serious problem. There are some plausible explanations for this.

- The antimalarial compound is labile under certain conditions (of temperature, acidity, basicity, light, solvent used, etc.) and progressive degradation occurs during the fractionation procedure.
- Some compounds are inactive on their own but may act synergistically with other constituents. When they become separated, antimalarial activity generally decreases or disappears. In this particular case, the crude extracts are far more active than the individual compounds.
- The fractionation procedure devised to isolate the bioactive constituents is inadequate.

16.6.2 Failure to Obtain Positive Results with Extracts Containing Antimalarial Constituents

Although some antimalarial plants do not display significant activities on either the erythrocytic or the hepatic stages, they may unexpectedly contain useful active compounds. Failure to identify these compounds could be explained as follows:

- The active compounds are present in insufficient quantity in the crude extract to display activity at the dose levels employed.
- If the bioactive compound is present in sufficient quantity, another explanation would be that other constituents may exert antagonistic effects during testing procedure, or some substances may stimulate some biological parameters that counteract the action of the bioactive compound, and therefore negate the effect of inhibitory constituents.
- The pharmacological model used to demonstrate the biological activity would be inappropriate. Some extracts are active *in vitro* but lack *in vivo* activity because bioactive constituents would be metabolised *in vivo* into inactive compounds, or they do not enter the parasite particularly because of their hydrophilic property. On the other hand, some extracts are active only *in vivo* because some components would be metabolised *in vivo* into active compounds.

16.6.3 False Positive Tests

One current false positive test is due to the erythrotoxicity of some constituents. This is the case of extracts containing saponins. Their strong *in vitro* activity is generally due to the known haemolytic action of some saponins, which affect the red blood cells.

16.6.4 Failure to Duplicate Antimalarial Results between Different Samples of the Same Plant

One explanation would be a variation in the concentration of active principles between samples as a result of environmental and genetic variability (period of collection, areas, developmental stages such as plant height, flowering time, size of vegetative features).

Another cause is the lack of ability to collect the same specimen on separate occasions (Rasoanaivo et al., 2002).

16.6.5 Discrepancies between Test Results for the Same Extract between Different Laboratories

Discrepancies have occurred in the results of *in vitro* antimalarial tests for the same extract between independent laboratories. Some discrepancies fortunately fall within the range of active extracts, which permits the bioassay-guided fractionation of the extracts. In other cases, however, extracts claimed to be active in one laboratory are found to be inactive in another and vice versa. This problem is still the subject of debate and controversies. Several parameters such as solvents used to dissolve the extracts (either dimethyl sulfoxide [DMSO] or methanol), parasitaemia, haematocrit, CO_2 concentration, stability of extracts, incubation time, and strains used may play an important role in the tests. Since it is practically impossible to harmonise all experimental procedures in various laboratories, discrepancies are unavoidable. The activity scaling proposed here for *in vitro* antiplasmodial activity should therefore be regarded as a flexible guide for selecting extracts for further investigation rather than a rigid rule to be followed in all cases.

16.7 SCENARIOS IN THE OUTCOME OF THE EFFICACY EVALUATION

There are several scenarios depending on the chemical structure of the bioactive individual compounds and their biological activity. The isolated antiplasmodial constituent may have:

- Both known structure and known antimalarial activity, i.e., isolating quinine in another plant species
- Known structure with antimalarial activity acting by a known validated mechanism
- Known structure with antimalarial activity acting by a novel mechanism
- New structure with antimalarial activity acting by a known validated mechanism
- Novel structure with antimalarial activity acting by a new mechanism

16.8 FURTHER INVESTIGATION OF ANTIMALARIAL PLANTS

Considering the international trend in malaria chemotherapy research, we have proposed in this paper two antimalarial screening targets, the erythrocytic and the hepatic stages of malaria parasites. However, unlike the tight selection of drug candidates for development in the pharmaceutical industry, the results should be handled with flexibility, taking into account the holistic approach of traditional medicine. There cannot be a rigid framework with defined decision points. The stages in the process of decision making toward a final product (efficacy evaluation, safety evaluation, standardisation, galenical formulation, clinical observation, and clinical trials) will be different for each plant that is investigated, and it is necessary to spend more time on the study of individual plants that have strong ethnobotanical evidence of usefulness in the treatment or prevention of malaria. Particularly, the high frequency of indications of a certain plant must encourage further insight into the study of antimalarial activity of a plant, with more appropriate methods. Several scenarios must be considered carefully before rejecting any extracts.

16.8.1 Extracts with Good *In Vitro* Potency but Lacking *In Vivo* Activity

This is sometimes encountered with herbal antimalarials. One explanation is that the active constituent(s) is metabolised *in vivo* into inactive compound(s). Another plausible explanation is the unsuitability of the *in vivo* rodent malaria models to demonstrate the expected activity. Additional *in vivo* models may be needed to adequately evaluate these antimalarial plants (Dow et al., 1999).

16.8.2 Extracts with Good *In Vivo* Activity but Lacking *In Vitro* Activity

To the best of our knowledge, this case is rare. One logical explanation is that constituents may act as pro-drugs, and *in vivo* metabolisation is required to yield active compounds. The cases of proguanil (Carrington et al., 1951; Crowther and Levi, 1953) and primaquine (Russell et al., 2003) illustrate this possibility. It is also possible that extracts act by an unknown or unexpected mechanism. The investigation of these extracts may open up new lines of research, possibly leading to the development of new tools for the treatment of malaria.

16.8.3 Extracts with neither *In Vitro* nor *In Vivo* Activity

Assuming that the plants are claimed to be efficient in treating malaria in a broad sense, there are many alternatives to deal with such a situation, and this has been explained in the integrated approach to antimalarial plants (Rasoanaivo et al., 2002). Particularly, the possible stimulation of the immune system of the infected host by antimalarial extracts is a relevant area to explore further (Foldes and Matyi, 1994; Murata et al., 1999). In some cases, successive treatments with various plants are frequently done until the patient is cured; thus, possible additive or synergistic activities should also be considered.

16.8.4 Phytomedicines with Several Plant Ingredients

Some antimalarial phytomedicines containing ingredients from several plants are used in primary health care (Gasquet et al., 1993). Each individual plant may have biological activities that fall into the categories discussed in this paper. It is also possible that some herbs have synergistic effects, and other materials may counteract the potential toxicity of other ingredients in the formulation. In our opinion clinical investigation should be tailored to adequately evaluate these traditional medicines and phytomedicines, and phytochemical studies from plant mixtures should not be discarded, but one must keep in mind that to isolate one or more active compounds from one plant is already difficult work; thus, to determinate interactions between a mixture of plants is even harder.

16.9 CONCLUSIONS

Because of the complex nature of biological systems, no kind of test can be expected to function perfectly. In other words, it is impossible to devise a test or series of tests that will identify all active substances with no false positives or false negatives. Therefore, scientists performing screening programs must show vigilance, ingenuity, imagination, and common sense in order to minimise the shortcomings of fractionation and tests so as not to allow true biological activity to go undetected. There is no one definitive or rigid scheme for efficacy evaluation of antimalarial plants; this is a dynamic process, and the rule adopted is learning by doing.

During the past 5 years, several screening programs of plant extracts for antiplasmodial activities have been published by or in collaboration with Third World scientists (Rasoanaivo et al., 2002; Antoun et al., 2001; Simonsen et al., 2001; Muñoz et al., 2000; Traore-Keita et al., 2000; Omulokoli et al., 1997). Furthermore, many active compounds have been discovered in laboratories based in developing countries. We believe that in the next decades to come, many useful antimalarial

compounds will be isolated from tropical plants. The majority of those discoveries will be made by or in collaboration with competent and highly motivated scientists in developing countries.

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