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The value of Central-African traditional medicine for lead finding: Some case studies

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Abstract

One of the possible methodologies for the discovery of novel drugs is the screening of selected plant extracts for a broad array of pharmacological activities. The selection based on enthnomedicinal uses, combined with a follow-up of existing literature on the plants' chemotaxonomic properties, would seem to be the most cost-effective strategy for finding active plant extracts. A bioassay-guided fractionation of the active extracts should subsequently lead to the isolation and identification of the active lead constituent(s). Taking into account the enormous number and the amazing structural diversity of the currently known plant constituents, one might hope that promising model compounds with new structures and/or novel mechanisms of action might be found. In order, however, to optimize such a natural product drug discovery methodology, dereplication and selectivity of activity should be included in the screening system. Dereplication by which known compounds can rapidly be identified from a partially purified mixture prevents a research group from wasting resources by rediscovering known compounds. The use of single-target specific bioassays such as tests on isolated enzymes or on receptor-binding, or multiple target functional bioassays on isolated organs or intact cells must allow at an early stage to isolate compounds with specific pharmacological properties. In this publication, several examples of bioassay-guided isolation and identification of pharmacologically active lead compounds from plants used in Central-African traditional medicine by our research group will be presented and discussed.

Keywords: Lead compounds; Medicinal plants; Central-African Traditional Medicine; Standardised extracts; Bioassay-guided Isolation; Pharmacognosy

1. Introduction

The term “pharmacognosy” as a constituent scientific discipline of pharmacy has been used for nearly 200 years, and it refers to studies on natural product drugs. During the last half of the 20th century, pharmacognosy evolved from being a descriptive botanical subject to one having a more chemical and biological focus. At the beginning of the 21st century, pharmacognosy teaching has been given new relevance as a result of the explosive growth in the use of herbal medicinal products (phytomedicines) in Western modern pharmacy practice. In turn, pharmacognosy research areas are continuing to expand and now include aspects of cell and molecular biology in relation to natural products, ethnopharmacology and phytotherapy, in addition to the more traditional analytical method development and phytochemistry (Kinghorn, 2001).

2. Drug discovery from natural products

Bioactive natural products have an enormous economic importance as specialty chemicals. They can be used as drugs, lead compounds, biological or pharmacological tools, feed-stock products (raw materials for the production of drugs), excipients and nutraceuticals. They are present in herbs, dietary supplements, spices and foods. Some of them are important flavours, fragrances, dyes and cosmetics, and others are used as insecticides, antifeedants, pesticides and rodenticides.

Nature only uses a few building blocks for its biosynthetic pathways, but the great diversity of the end products results from various “decorating” enzymes found in each species, that introduce new functionalities. Combinatorial chemistry has even been called “the chemist’s surrogate for the rain forest”. When compared with libraries of synthetic substances, natural products offer the prospects of discovering a greater number of compounds, with sterically more complex structures (Hogan, 1997; Henkel et al., 1999).

About 155,000 seed plants occur in the tropics with about 120,000 in the tropical moist forest alone (only 7-8% of the land surface on earth). This is due to the high humidity, elevated temperature, species density and a continuous growing season. The result is an enormous diversity of chemical structures, which are not waste products, but specialised

secondary metabolites involved in the relationship of the organism with the environment, e.g., as attractants of pollinators, signal products, defensive substances against predators and parasites, or in resistance against pests and diseases. Since only 5-15% of the higher plants have been systematically investigated for the presence of bioactive compounds, nature's biodiversity remains largely unexplored. This is even more true for other sources from nature such as marine organisms, microorganisms and animals (Cragg et al., 1997; Newman and Cragg, 2012).

Different approaches to drug discovery using higher plants can be distinguished: random selection followed by chemical screening; random selection followed by one or more biological assays ; follow-up of biological activity reports (e.g., ecology based); follow-up of ethnobotanical (traditional medicine) use of plants. The latter approach includes plants used in organised traditional medical systems; herbalism, folklore and shamanism ; the use of databases (Fabricant and Farnsworth, 2001). The objective is the targeted isolation of new bioactive plant products, i.e., lead substances with novel structures and novel mechanisms of action.

Several "drug discovery hurdles" have to be taken. When an active extract has been identified, the first hurdle to be taken is dereplication (the rapid identification of known compounds from a partially purified mixture, and identification of enough of an unknown structure to prioritise or conduct an isolation procedure). This can mean either a full identification of a natural product after only partial purification, or partial identification to the level of a family of known compounds. Using "hyphenated" or "tandem" techniques separation is carried out with the components analysed "online" by one or more spectroscopic methods. The second hurdle is purification of the new compound by chromatographic methods, the third hurdle is structure determination by spectroscopic methods, and when the novel structure has been established, the fourth hurdle is scale-up of the production. The fifth and final hurdle is the medicinal chemistry phase, and when an optimised lead compound has been characterised the drug development process can start. Testing of large numbers of compounds or extracts in an automated fashion will increase the number of molecules entering the drug development process. High-throughput screening (HTS) uses single-target specific bioassays (tests on isolated enzymes or on receptor binding) or multiple target functional bioassays (assays on whole animals, isolated organs or intact

cells). Bioassays can be miniaturised in high-density micro-well plates (e.g. 1536-well plates) at microliter scale (volumes of 1-2 µl).

There are some general requirements for screening bioassays of plant extracts : validity, lack of ambiguity, accuracy, reproducibility, simplicity and a reasonable cost. Some special considerations include a high selectivity (to limit the number of leads for follow-up evaluation), a high sensitivity (to detect low concentrations of active compounds), and a high specificity (to be insensitive to a wide variety of inactive compounds) (elimination of false positives). The methodology should also be adaptable to highly colored, tarry, poorly water soluble and chemically complex materials.

In Figure 1, a flow chart is presented for the study of plants used in traditional medicine. In a "normal" drug discovery process pure active compounds, obtained by a bioassay-guided isolation from extracts of medicinal plants, are subjected to structure-activity relationshop studies (SAR). Toxicity and safety studies as well as clinical tests are carried out, active compounds have to be prepared on a industrial scale, and an appropriate pharmaceutical formulation has to be developed before the compound can be approved as a drug. In a "traditional" medicine system, however, pharmacological evaluation of extracts from medicinal plants may lead to the establishment of standardised extracts. In this case, the industrial production of these standardised extracts can start immediately after toxicity and safety studies. After formulation of the standardised extracts clinical tests can be carried out, which may lead to approval as drugs.

(Fig. 1)

Taking everything mentioned above into account, it is obvious that the investigation of medicinal plants is a multidisciplinary process. Figure 2 shows an example of how a multidisciplinary research team could be composed and which interactions between the members are needed. The pharmacognosist has to interact closely with a systematic botanist, by preference affiliated to a university or a research institute in the country of origin of the plant material, for the collection and identification of the plant material. If the botanist is an ethnobtanist, he will also be able to provide or to collect ethnobotanical and ethnopharmacological information. Extraction, isolation and structure elucidation have to be carried out in the pharmacognosist's laboratory, in close cooperation with a

pharmacological, microbiological or biochemical unit, depending on the type of bioassays used for the *in vitro* screening of plant extracts, the bioassay-guided fractionation, and the *in vivo* screening. In case a detailed investigation of structure-activity relationship is needed, a medicinal chemist will have to synthesise analogues and derivatives, but this approach may be limited to compounds or compound classes accessible for organic synthesis.

(Fig. 2)

3. The value of traditional medicines

Early people confronted with illness and disease discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and books. Many valuable drugs of today e.g. morphine, digoxin, quinine, tubocurarine and many others came into use through the study of endogenous remedies.

In the new millennium ethnopharmacology i.e. the scientific study of traditional medicines used by different cultural groups has become an important discipline at many universities. Also health authorities such as the World Health Organisation (WHO) and the European Medicines Agency (EMA), promote the preservation of local knowledge on traditional medical systems such as Kampo, traditional Chinese Medicine, Unani, Ayurveda etc… and the corresponding traditional drugs in primary health care and the conservation of biodiversity. In the current legal framework of the European Union herbal medicinal products (HMPs) can obtain a regular marketing authorisation as full drugs or drugs based on a well-established use or they can be registered as traditional drugs. It is therefore not necessary anymore to isolate the active ingredients from a herb and market them in a highly purified form. Total extracts of a single herb or mixtures of different herbs in a formulation can be used, if a chemical profiling and a quantitative standardisation of the herbal medicinal product are carried out. Nowadays, extensive systematic analysis of metabolites in HMPs is available by using high-resolution nuclear magnetic resonance (NMR) spectroscopy. In combination with additional techniques, primarily mass spectrometry (MS) and

chromatographic methods and with support of involved statistical and other data analysis tools, NMR offers an outstanding tool for qualitative and quantitative analysis and risk assessment of HMPs (Pelczer, 2005). The inclusion of such phytomedicines in the current armamentarium of Western drugs creates new opportunities for teaching pharmacognosy and/or phytotherapy at the faculties of pharmacy and medicine at the Western universities.

4. Examples of lead finding from Central-African traditional medicine by our research group

As already pointed out our multidisciplinary team of the University of Antwerp has been working for more than thirty years according to the above described methodology (see Figure 1). Without trying to be complete, Table 1 gives a list of some successful examples of this approach in our team, where lead compounds have been isolated from Central-African medicinal plants, and in some cases used as starting point for the synthesis of analogues or derivatives in order to study structure-activity relationships, and to optimize the activity (Pieters and Vlietinck, 2005). The last example even led to successful clinical studies of a standardised extract developed from the investigated medicinal plant (Mesia *et al*., 2012a, 2012b).

(Table 1)

5. Examples of lead finding worked out in detail

5.1. *Euphorbia grantii* Oliv. and related *Euphorbia* spp. (Euphorbiaceae) and common cold

Although in many countries the name *Euphorbia* is considered as synonymous with poison, nevertheless many *Euphorbia* sp. are used for a variety of medicinal purposes, including the treatment of infections and childhood diseases among them also poliomyelitis. An investigation of the constituents responsible for the pronounced antiviral activity observed for extracts of *Euphorbia grantii* Oliv. (Ruanda) and *E. balsamifera* Ait. (Senegal) afforded seven related 3-*O*-methylquercetin (3-MQ) and 3-*O*-methylkaempferol (3-MK) flavones exhibiting remarkable *in vitro* activities against picornaviruses, including poliomyelitis-,

coxsackie- and rhinoviruses and vesicular stomatitis virus (Figure 3). When administered intraperitoneally, 3-MQ protected mice from viraemia and lethal infections from coxsackie B_4 virus at a daily dose of 20 mg/kg for a period of nine days, whereas no signs of toxicity or death were observed in uninfected control mice, that were treated with five times the level of 3-MQ used to treat infection mice (Van Hoof et al., 1984).

(Fig. 3)

Studies on the mechanism of action of 3-MQ on poliovirus replication showed that these compounds interfere with an early stage in viral synthesis. In a detailed investigation it was found that there is a narrow window between 1 and 2.30 h post infection during which 3-methoxyflavones exert their action and succeed in blocking virus replication. It was also demonstrated that during that period the polio-induced shut-off of cellular protein synthesis persisted even when the compound concentration was high enough to completely inhibit viral RNA and protein synthesis. This suggested that the viral factor that caused the shut-off did not depend on the *de novo* protein synthesis (Vrijsen et al., 1987). Although the exact mode of action is not completely understood yet, it has been found that 3-MQ inhibits the formation of both minus- and plus-strand viral RNA of poliovirus by interacting with the proteins involved in the binding of the virus replication complex to vesicular membranes where poliovirus replication taken place (Castrillo and Carrasco, 1987). Structure-activity studies of naturally occurring flavones indicated that the 3-methoxyl- and the 4'-hydroxylfunctions were essential for the antipicornaviral activity. Therefore, a series of A-ring substituted (methyl, hydroxy, methoxy, halo, amino, nitro)-4'-hydroxy-3-methoxyflavones were synthesized and antivirally tested against polio- and rhinoviruses. In this series, 4',7-dihydroxy-3-methoxy-5,6-dimethylflavone was the most active compound, possessing *in vitro* TI_{90}-values of > 1000 and > 200 against poliovirus type 1 and rhinovirus type 15 respectively. This compound was also active against all other rhinovirus serotypes tested, having MIC_{50} values ranging from 0.016 to 0.5 µg/ml. In contrast to quercetin it showed not to be mutagenic in the Ames test (Demeyer et al., 1991) (Figure 4).

(Fig. 4)

Since human rhinoviruses are one of the major causes of common cold (30-50%) leading to manifestations of illness including rhinorrhea, cough, sneezing and sore throat and to nasal obstruction and increased mucus production, the finding of drugs to prevent or treat common cold are most beneficial.

A second class of natural compounds consisting of flavans and chalcones and several synthetic compounds developed by pharmaceutical companies as potential antirhinovirus drugs such as pyridazines, thiazoles and methyl-isoxazoles inactivate rhinoviruses directly by binding to or interacting with specific sites on the rhinovirus capsid proteins. These capsid binding compounds have prominent antirhinovirus properties, but also a substantial serotype variability leading to substantial *de novo* resistance (Andries et al., 1991) (Figure 5).

(Fig. 5)

In order to combine the antiviral properties of both classes of antirhinovirus substances, hybrid molecules were synthesized consisting of a 3-methoxyflavone nucleus, which was coupled at the 7-0-position via an alkyl-side chain with a capsid-binding compound such as 3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]pyridazine (Boers et al., 1998) (Figure 6).

(Fig. 6)

These hybrid compounds block sequentially two consecutive steps of rhinovirus replication and consequently exclude *de novo* resistance or the development of resistant rhinovirus strains.

Finally, anti-inflammatory properties of 3-methoxyflavones have been shown, which might be beneficial for the treatment of several symptoms of common cold due to the release of inflammatory mediators such as kinins following a viral infection of the nasal cells. Also formulation studies have been implemented in order to improve the bioavailability of the compound into nasal cells.

In summary, in this example of lead finding a new class of compounds with pronounced antiviral activities against picornaviruses, an attractive new mechanism of action and a lack

of resistance induction was developed from a traditional African drug used against infectious diseases, among them poliomyelitis.

5.2. *Cryptolepis sanguinolenta* (Lindl.) Schlechter (*Periplocaceae)* and malaria

Aqueous decoctions or macerates of the root or the root bark of the climbing liana *Cryptolepis sanguinolenta* (Lindl.) Schlechter (Periplocaceae) are used in traditional medicine in Central and West Africa for the treatment of infectious diseases, amoebiasis and fevers, including fever by malaria. Until 1990, only two alkaloids had been characterised from this plant : cryptolepine, the major alkaloid and its analogue, quindoline, which lacks the N-methylgroup. Both alkaloids are indolo[3,2-b]quinolines (benzo-δ-carbolines). Antiplasmodial properties *in vitro* against chloroquine-sensitive *Plasmoduim falciparum* (D-6 strain) and chloroquine-resistant K-1 and W-2 strains as well as *in vivo* activities in mice infected with *P. berghei berghei* and *P. berghei yoelii* were found for cryptolepine but much less for quindoline. This already demonstrated the importance of the N-methylgroup for antimalarial activity of these alkaloids (Cimanga et al., 1997). During the past decades, a whole series of minor alkaloids have been isolated from *Cryptolepis sanguinolenta*, among them isocryptolepine (a benzo-δ-carboline), neocryptolepine (a benzo-α-carboline) and the dimeric alkaloids named biscryptolepine and cryptoquinoline (Cimanga et al., 1996a). In addition to the antimalarial activity cryptolepine and analogues showed antibacterial but no antifungal activities (Cimanga et al., 1996b, 1998) (Figure 7).

(Fig. 7)

A variety of diverse pharmacological effects have also been reported for cryptolepine *viz*. hypotensive and antipyretic effects, presynaptic α-adrenoreceptor blocking action, antimuscarinic properties and antiinflammatory properties. In addition cryptolepine displays cytotoxic activities against tumour cells for example it efficiently inhibitis the growth of B16 melonama cells. Therefore the mechanism of action of the latter was investigated. These studies revealed that cryptolepine and neocryptolepine can intercalate DNA cleavage by human topoisomerase II. The two alkaloids interfere with the catalytic activity of human

topoisomerase II but the poisoning activity is slightly more pronounced with cryptolepine than with its isomer, indicating a reduced cytotoxicity of neocryptolepine compared with the parent molecule (Bailly et al., 2000). Therefore, neocryptolepine was selected as a lead for the development of new antimalarial compounds. A series of 2- and 9 substituted as well as 1- and 3-substituted derivatives were synthetized and structure-activity studies were carried out using chloroquine-sensitive as well as chloroquine-resistant *P. falciparum* strains. All compounds were also tested for their activity against *Trypanosoma brucei* and *T. cruzi* and for their cytotoxicity on human MRC-5 cells. Mechanisms of action were investigated by testing haeme-complexation using ESI-MS, inhibition of β-haematin formation (BHIA), DNA interactions (DNA-methylgreen assay and linear dichroism), and inhibition of human topoisomerase II (Jonckers et al., 2002).

The neocryptolepine derivatives can be classified in different groups (Table 2). A first group consists of compounds with a selective antiplasmodial activity, which are able to inhibit the formation of β-haematin ; therefore their mechanism of action is most probably related to inhibition of the haeme detoxification process. The most prominent example of this group is 2-bromoneocryptolepine. On the other hand, a second group consists of compounds showing a non-selective antiplasmodial activity, which is related to DNA-interactions, but which are not capable of inhibiting the formation of β-haematin. A typical compound in this group is 2-methoxyneocryptolepine. Finally, a third group of compounds contain antiplasmodial agents with a mixed mechanism of action, such as neocryptolepine and cryptolepine. All these compounds lack selectivity towards the *Plasmodium* parasite.

(Table 2)

Although 2-bromoneocryptolepine can be considered as the most promising lead in this series of the neocryptolepine derivatives, a further optimalisation of both the antiplasmodial activity and the selectivity is needed before a potentially clinically useful therapeutic agent may be obtained (Van Miert et al., 2004, 2005a).

Because of the promising properties of these indoloquinolines, it was decided to synthetise the missing isomer in this quartet of indoquinolines i.e. the corresponding benzo-β-carboline, being an indolo[2,3-c]-quinoline derivative, to compare its biological activities

with the naturally occurring isomers. This compound, for which we have adopted the name isoneocryptolepine, has not been reported from nature (Figure 8).

(Fig. 8)

The antiprotozoal activities of the three naturally occurring isomeric indoloquinoline alkaloids i.e. **(1)** cryptolepine, **(2)** neocryptolepine and **(3)** isocryptolepine and the two dimeric indoquinoline alkaloids **(6)** cryptoquinoline and **(7)** biscryptolepine, originally obtained from *Cryptolepis sanguinolenta*, were compared with those of the synthetic isomer, **(4)** isoneocryptolepine and a quartenary derivative, **(5)** N-methyl-isocryptolepinium iodide (Table 3). The latter compounds showed a high antiplasmodial activity against the chloroquine-resistant *Plasmodium falciparum* strain K1 (IC_{50} of 0.23 µM and 0.017 µM, respectively), while the cytotoxicity was 4.32 and 12.7 µM respectively. Isoneocryptolepine was found to act as an inhibitor of β-haematin formation and as a DNA-intercalating agent (Van Miert, 2005b) (Table 4).

(Table 3)
(Table 4)

Later on a series of chloro- and aminoalkylamino-substituted neocryptolepine (5-methyl-5*H*-indolo[2,3-*b*]quinoline) derivatives were synthesized and evaluated as antiplasmodial agents. The evaluation also included cytotoxicity (MRC5 cells), inhibition of *β*-hematin formation, and DNA interactions (DNA-methyl green assay). Introduction of aminoalkylamino chains increased the antiplasmodial activity of the neocryptolepine core substantially. The most efficient compounds showed antiplasmodial activities in the nanomolar range. N^1,N^1-Diethyl-N^4-(5-methyl-5*H*-indolo[2,3-*b*]quinolin-8-yl)pentane-1,4-diamine showed an IC_{50} of 0.01 μM and a selectivity index of 1800 (Fig. 9) (El Sayed et al., 2009).

(Fig. 9)

In summary, in this example of lead finding the known antiplasmodial activity of cryptolepine was extended to the three other isomers of the indoloquinoline alkaloids and their mechanism of action was investigated. The antiplasmodial activity of these alkaloids is due to a combination of at least two mechanisms of action. Inhibition of the haeme detoxification process is a selective mechanism, whereas DNA intercalation, a non-selective mechanism, is responsible for the cytotoxicity and probably also for the activity against other parasites tested.

5.3. *Nauclea pobeguinii* (Pob. Ex Pell.) Petit (Rubiaceae) and malaria

In 1998 an ethnobotanical survey was carried out on plants used to treat presumed malaria or malaria-like symptoms in some provinces of the DR Congo, and indicated that an aqueous decoction of the stem bark of *Nauclea pobeguinii* (Pob. ex. Pell) Petit (Rubiaceae) was most frequently used for this purpose in the Sankuru District (Province of Kasai-Oriental) (Mesia et al., 2005). Phytochemical investigations have revealed the presence of the alkaloid strictosamide as the main constituent of this plant, besides other minor indole alkaloids and some quinovic acid glycosides (Zeches et al., 1985, Anam, 1997). From the 80% ethanol extract, 5 known compounds were isolated and identified as (5S)-5-carboxystrictosidine, 9-O-methylangustoline, 3-O-β-fucosyl-quinovic acid, 3-ketoquinovic acid and strictosamide (Fig. 10).

(Fig. 10)

Because of its widespread use against malaria in the DR Congo, a HPLC-method for the determination of alkaloids in *Nauclea pobeguinii* preparations was developed and validated, in order to allow further biological, pharmacological and clinical investigations. Since strictosamide was characterised as the active principle from *Sarocephalus latifolius* (*Nauclea latifolia)* showing IC_{50} values against *Plasmodium falciparum* K1 and NF54 of 0.45 and 037µg/mL (Abreu and Pereira, 2001), it was decided to standardise *Nauclea pobeguinii* extracts on strictosamide (Dhooghe et al., 2008).This extract was used for *in vitro* evaluation

of antiplasmodial activity and cytotoxicity, *in vivo* evaluation of antimalarial activity, and acute and sub-acute toxicity in laboratory animals (Mesia et al., 2010).

The aqueous and 80% EtOH extracts from *N. pobeguinii* stem bark, and its constituents were evaluated for their *in vitro* activity against *Plasmodium falciparum* (chloroquine-sensitive Ghana-strain). The 80% EtOH extracts, containing 5.6% strictosamide, was evaluated *in vivo* in the 4-day *P. berghei* mouse model, and in the *P. yoelii* N67 model. All compounds were inactive or only moderately active *in vitro*. The aqueous and 80% EtOH extracts displayed moderate *in vitro* activity with IC_{50} values of 44 and 32 µg/mL, respectively, without apparent cytotoxicity on MRC-5 cells (CC50>64 µg/mL). Daily oral dosing ot he 80% EtOH extract, at 300 mg/kg, resulted in 86% reduction of parasitaemia in the 4-day *P. berghei* mouse model, and 75% reduction in the *P. yoelii* N67 model. Prolonging oral dosing to 2 x 5 days, with an interval of 2 days, and oral administration of the 80% EtOH extract at 300 mg/kg induced 92% reduction of parasitaemia, and a mean survival time of 17 days. Strictosamide, the putative active constituent, may be metabolically activated in the gastrointestinal tract after oral administration. Levels of creatinine, urea, ALAT and ASAT (alanine- and aspartate-aminotransferases) remained unchanged after treatment. No acute toxicity was observed in mice after a single 2g/kg oral dose, nor after 4 weekly doses. No significant macroscopic or microscopic lesions were observed in heart, lung, spleen, kidney, liver, large intestine and brain. It was concluded that: these results can partly support and justify the use of *N. pobeguinii* in traditional medicine in the DR Congo for the treatment of uncomplicated malaria.

Therefore, it was decided to carry out a clinical phase I study using the quantified 80% ethanol extract from the stem bark of *Nauclea pobeguinii* (PR 259 CT1) in order to assess its short term safety and tolerability in healthy adult male volunteers (Mesia et al., 2011).The amount of the major alkaloid strictosamide in the 80% ethanol extract was determined by a validated HPLC method and was shown to be 5.6%. The herbal preparation was formulated in a gelatine capsule form containing 500 mg of PCR 259 CT1. A sample of 15 healthy male volunteers, selected using the Lot Quality Assurance of Sampling (LQAS) method, was eligible for inclusion after fulfilment of the inclusion criteria and clinical examination by a physician. The volunteers were treated in an out-patient clinic with a drug regimen of two 500mg capsules three times daily (each eight hours) for seven days, during meals. This drug regimen

was obtained by mathematical conversion of animal doses obtained in several *in vivo* studies in mice to human equivalent doses as in falciparum malaria patients.

Safety and tolerability were monitored clinically, haematologically, biochemically and by electrocardiographic (ECG) examination at days 0, 1, 3, 7 and 14. Adverse effects were recorded by self-reporting of the participants or by detection of abnormalities in clinical examinations by a physician. The oral administration of PR 259 CT1 at high doses of 2x 500mg/capsule/day for 7 days was found to induce no significant changes in the concentration levels of all investigated haematological, biochemical, electrocardiogram and vital sign parameters and physical characteristics after 14 days of treatment compared to those seen in the baseline data. The concentration levels of all evaluated parameters were within the normal limits as reported in the literature. All adverse events noted were mild and self-resolving including increase of appetite (33%), headache (20%) and nausea (20%). Other minor side effects were insomnia, somnolence and asthenia (7%). Thus, PR 259 CT1 presented a significant safety and tolerability in healthy volunteers to allows its further development by starting a phase II clinical trial.

Phase II clinical trials are designed to assess how well a drug works as well as to continue phase I safety assessments in a larger group of volunteers and patients. Phase II studies are sometimes divided into phase IIA and phase IIB. The first one is specially designed to evaluate dosing requirements (how much should be given), whereas the latter is specifically designed to study efficacy [how well the drug acts at the prescribed dose(s)].

The aim of this phase IIA clinical trial was to assess the efficacy of PR 259 CT1 in a small group of adult patients diagnosed with uncomplicated falciparum malaria. The phase IIA study was an open cohort study in eleven appraisable adult patients suffering from proven *Plasmodium falciparum* malaria. The study was specifically designed to assess the efficacy of PR 259 CT1 administered with a dose regimen of two 500mg capsules three times daily for three days, followed by out-patient treatment of one 500mg capsule three times daily for the next four days, in order to prove that this therapeutic dose, which was calculated from animal doses, was effective to treat adult malaria patients and consequently useful for a future Phase IIB clinical trial. This study would then substitute a dose-escalating trial, which in general is used to find the appropriate dose for clinical studies. The phase IIA clinical trial was carried out according to the WHO 2003 14-days test, and the results revealed that all eleven patients were completely cleared of parasitaemia and fever on days 3, 7, and 14

except for one patient, who experienced a recurrence of parasitaemia at days 7 until 14. Besides this adequate clinical and parasitological response (ACPR), this trial also demonstrated that PR 259 CT1 was well tolerated with only mild and self-resolving adverse effects including fatigue and headache, which were in accordance with those found in the phase I clinical trial. Moreover, all symptoms progressively disappeared, and no symptoms were observed on day 14. Although the number of patients included in this study was rather limited, the statistical analysis nevertheless suggested the efficacy and tolerability of PR 259 CT1, which indicated that this herbal medicinal product might be considered as a putative candidate for a large scale clinical trial (Mesia et al., 2012a).

According to the promising results of the Phase I and Phase IIA clinical trials with the herbal medicinal product PR 259 CT1, a Phase IIB study was conducted as a single blind prospective trial in 65 patients with proven *Plasmodium falciparum* malaria to evaluate the effectiveness and safety of this herbal drug (Mesia et al., 2012b). The study was carried out simultaneously using an artesunate-amodiaquine combination (AS +AQ) (Coaresucam ®) as a positive control. This combination is the standard first-line treatment for uncomplicated malaria recommended by the National Programme of Malaria Control in the DR Congo. With regard to PR 259 CT1, patients were treated with a drug regimen of two 500-mg capsules three times daily for three days in the in-patient clinic, followed by out-patient treatment of one 500-mg capsule three times daily during the next four days; the positive control group received two tablets containing 100 mg artesunate and 270 mg amodiaquine (fixed-dose) once daily during three consecutive days. Antimalarial responses were evaluated according the WHO 2003 guideline for a 14-day test. The results from the physical and laboratory examinations did not show any significant changes in values of vital signs, ECG, biochemical, and haematological parameters. The clinical and parasitological results of the study showed a rapid decline in the level of parasitaemia and no statistically significant difference in daily mean parasitic counts between the PR 259 CT1-treated patients (n=33) and the AS + AQ-treated patients (n=32). However, no statistical methods were used to calculate the sample sized needed to show a difference between the two treatments. Nevertheless, ACPR which was the primary outcome measure was seen in 87.9% of PR 259 CT1-treated patients compared to 96.6% of the AS + AQ-treated patients. There were 3.0% of ETF and 6.1% of LCF in the PR 259 CT1 group, none in AS + AQ group. Interestingly, one PCR-unadjusted LPF (3.0%) occurred in each group (Table 5).

(Table 5)

Referring to the pharmacokinetics of artesunate and amodiaquine, the first cited is rapidly converted in the organism to its main active metabolite dihydroartemisinin and rapidly cleared (elimination half-life generally < 1h). Oral amodiaquine is rapidly absorbed and undergoes rapid and extensive metabolisation to desethylamodiaquine, the main active metabolite. Amodiaquine has a short elimination half-life (approximately 5h), while that of desethylamodiaquine is much longer (6-18 days). Even though their combination was reported to reduce the total drug exposure, the recurrence that occurred within eleven days after the end of treatment in the AS + AQ group is more likely to be recrudescence than reinfection. The results of the study showed no significant difference in FCT between patients receiving AS + AQ and those receiving PC 259 CT1 and a more rapid parasite clearance by patients receiving AS + AQ (Fig 11).

(Fig. 11)

The two regimens used were found to be safe in the study; no life-threatening clinical or biological adverse events were observed and all mild adverse events resolved spontaneously. Patients treated with AS + AQ were more likely to develop side effects than PR 259 CT1-treated patients. The most frequent adverse events in the AS + AQ group were asthenia, dizziness, pruritus, and anorexia as previously reported in several recent studies (Brasseur et al., 2007; Maiztiki-Sebagazi et al., 2008).

In conclusion, PR 259 CT1 was assessed for its antimalarial afficacy and safety in a Phase IIB trial in comparison with artesunate-amodiaquine in patients infected with non-severe malaria. Evaluated on the evidence of fever clearance, disappearance of parasitaemia, and other symptoms, as well as adequate clinical and parasitological responses (ACPR) according to the WHO 2003 criteria, both treatments have been shown to be effective in the treatment of uncomplicated malaria in adult patients. AS + AQ appeared to be slightly more effective while PR 259 CT1 was better tolerated. The trial was done in adults living in areas of high transmission. Therefore, the results must be interpreted in the context of the age of the patients taking into consideration a possible synergy between drug treatment and acquired immunity. The results are sufficient, according to the recommendation of the guidelines for

clinical study on herbal antimalarials, to endorse the local use on of PR 259 CT1 in an area where the remedy has traditionally been used. On the other hand, future trials considering combinations of *N. pobeguinii* either with other antimalarial drugs or with other antimalarial plants, with the aim of improving the efficacy and reducing recrudescence rates should be envisaged as well.

6. Conclusion

From Table 1 and the three examples of leadfinding worked out in detail, it can be stated that the major activities of natural products research of the past decades in our laboratory have clearly demonstrated that natural products represent an unparalleled reservoir of molecular diversity to drug discovery and development, and are complementary to combinatorial libraries. The major disadvantage is the time taken to isolate and to characterise the active components from the extracts. By improving diversity and quality of sample source and screen suitability, by accelerating dereplication and by automating and standardising early isolation steps, the effectiveness of natural products research can be enhanced.

For developing countries, the approval as drugs of standardised and formulated plant extracts might be the starting point of an innovative and successful local pharmaceutical industry, which can compete with the Western pharmaceutical companies, not only for the treatment of minor diseases, but also for the treatment of severe and life-threatening diseases.

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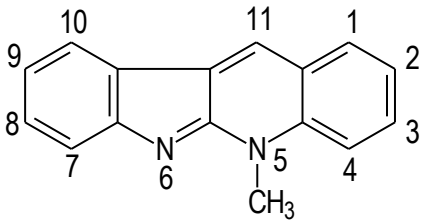
Table 1. Some lead compounds isolated from African medicinal plants in our research group at the University of Antwerp

Medicinal Plant	Chemical class of lead	Bioactivity
Annona muricata L.	*Alkaloids*	*Serotonine-like*
Apodytes dimidiata (E. Mey ex. Arn)	*Saponins*	*Antiprotozoal, antiangiogenic*
Borreria verticillata (L.) G.F.W. Mey	*Alkaloids*	*Antibacterial, antiangiogenic*
Bridelia ferruginea Benth.	*Flavonoids*	*Anticomplement, antioxidant*
	Caffeoylesters	*Antiviral*
Crataegus sinaica Boiss.	*Flavonoids*	*Antioxidant, anticomplement*
Crypotolepis sanguinolenta (Lindl.) Schlechter	*Alkaloids*	*Antibacterial, antifungal, antiparasitic*
Desmodium adscendens (Sw.) D.C.	*Cyclitol*	*Liver protection*
Euphorbia sp.	*3-O-Methylated flavonols*	*Antiviral*
Garcinia huillensis WeIw. ex Oliv.	*Polyisoprenylated benzophenones*	*Antibacterial, antifungal*
Harrisonia abyssinica Oliv.	*Prenylated polyketides*	*Antifugal, antibacterial, antiviral, molluscicidal*
	Limonoids	
	Chromones	
	Steroids	
Maesa lanceolata Forsskal. var. g*olungensis*	*Saponins*	*Antifugal, virucidal, molluscicidal, antiangiogenic*
Morinda morindoides (Baker) Milne-Redhead	*Flavonoids*	*Antioxidant, anticomplement*
	Anthraquinones	*Antiparasitic*
Nauclea pobeguinii (Pob. ex. pell.) Petit	*Alkaloids*	*Antimalaria*
Omecarpum kirkii (S. Moore)	*Biflavonoids*	*Antimalaria*
Pavetta owariensis P. Beauv.	*Condensed tannins*	*Antiviral, schistosomicidal immunomodulating*
Pterotaberna inconspicua Stapf.	*Alkaloids*	*Anticholinergic, anthihistaminic*
Quassia africana Baill.	*Quassinoids*	*Antiviral*
Spathodea campanulata Beauv.	*Polysaccharides*	*Antidiabetic, wound-healing*
Spondias mombin L.	*Hydrolysable tannins*	*Antiviral*
	Caffeoyl esters	*Antiviral*
	Alkenyl phenols	*Molluscicidal, insecticidal*
Tetradenia riparia (Hochst) Codd.	*Essential Oil*	*Antibacterial, antifungal*
	Diterpenes	*Antibacterial, antifungal*
	α-Pyrones	*Cytotoxic*
Vernonia amygdalina Del.	*Sesquiterpene lactones*	*Antiplatelet aggregation, antimalaria*
Zizyphus spina-christi L.	*Flavonoids*	*Antiviral, antioxidant*

Table 2. Antiplasmodial activity, cytotoxicity, DNA-methylgreen assay and inhibition of β-haematin formation of cryptolepine, neocryptolepine and synthetic deivatives



Neocryptolepine

	P. falciparum W2, Chloroquine resistant (IC_{50}, μM)	Cytotoxicity (MRC-5 cells) (IC_{50}, μM)	DNA intercalation (IC_{50}, μM)	BHIA assay (IC_{50}, Meq)
Cryptolepine	2.0 ± 0.1	1.5 ± 0.7	65.7 ± 3.0	17.2 ± 0.36
Neocryptolepine	14.0 ± 1.7	11.0 ± 1.4	92.8 ± 9.7	5.97 ± 0.22
2-Me-neocryptolepne	2.3 ± 0.6	0.95 ± 0.07	(not tested)	(not tested)
2-Br-neocryptolepine	4.0 ± 0.1	> 32	> 400	1.77 ± 0.15
2-OMe-neocryptolepine	4.7 ± 0.6	4.0 ± 0.1	77.9 ± 4.4	No inhibition
1-Br-neocryptolepine	> 32	16.0 ± 0.1	> 400	No inhibition
3-Br-neocryptolepine	4.7 ± 0.6	18.5 ± 0.7	> 400	2.56 ± 0.18

Table 3. Antiprotozoal activity and cytotoxicity of cryptolepine and related compounds

Compound	*Trypanosoma brucei rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani*	*Plasmodium Falciparum* (K1)	Cytotoxicity (L6 cells)
(1) cryptolepine	0.60 ± 0.07	0.22 ± 0.07	2.68 ± 0.89	0.12 ± 0.02	1.12 ± 0.07
(2) neocryptolepine	2.23 ± 082	2.01 ± 1.30	49.5 ± 3.7	2.61 ± 0.67	3.24 ± 0.04
(3) isocryptolepine	0.52 ± 0.11	1.27 ± 0.78	39.1 ± 11.5	0.78 ± 0.30	1.19 ± 0.26
(4) isoneo-cryptolepine	6.48 ± 0.067	21.0 ± 0.2	75.4 ± 2.4	0.23 ± 0.04	4.32 ± 0.04
(5) N-methyl-isocryptolepinium iodide	0.37 ± 0.21	236.10	1.15	0.017 ± 0.004	12.7 ± 2.0
(6) cryptoquinoline	96.4 ± 31.0	84.4 ± 35.6	> 65	> 10	165.4 ± 42.6
(7) biscryptolepine	2.57 ± 0.47	3.48 ± 1.48	8.93	0.27 ± 0.02	13.62
Melarsoprol[a]	0.0038 ± 0.0023				
Benznidazole[a]		1.50 ± 0.58			
Miltefosine[a]			0.58 ± 0.07		
Chloroquine[a]				0.17 ± 0.05	
Artemisinin[a]				0.042 ± 0.002	
Podophyllotoxin[a]					0.018

[a]: positive control

Table 4. Antiplasmodial activity, cytotoxicity, haeme-binding activity and DNA-intercalation of *Cryptolepis* alkaloids and derivatives

Compound	*Plasmodium falciparum* (K1) (IC_{50})	Cytotoxicity (L6 cells)	BHA (IC_{50})[b]	DNA intercalation (IC_{50})[c]
(1) cryptolepine	0.12 ± 0.02	1.12 ± 0.07	1.72 ± 0.36	65 ± 3
(2) neocryptolepine	2.61 ± 0.67	3.24 ± 0.04	5.97 ± 0.22	93 ± 10
(3) iso-cryptolepine	0.78 ± 0.30	1.19 ± 0.25	7.59 ± 0.34	118 ± 25
(4) isoneo-cryptolepine	0.23 ± 0.04	4.32 ± 0.04	5.24 ± 0.23	124 ± 19
(5) N-methyl-isocryptolepinium iodide	0.017 ± 0.004	12.7 ± 2.0	> 13[d]	Not tested
(6) crypto-quinoline	> 10	165.4 ± 42.6	2.56 ± 0.31	Not tested
(7) bis-cryptolepine	0.27 ± 0.02	13.62	7.40 ± 0.72	> 360
Chloroquine[a]	0.17 ± 0.05			
Artemisin[a]	0.0.42 ± 0.002			
Podophyllotoxin[a]		0.018		

[a] : positive control

[b] : IC_{50} represents the molar equivalents of test compounds, relative to haemin, required to inhibit β-haematin formation by 50%

[c] : IC_{50} expressed in µM

[d] : no inhibition observed at the highest test concentration (13 Meq)

Table 5. Clinical and parasitological response at day 14 according to treatment group

Endpoint	AS + AQ (n=32)		PR 259 CT1 (n=33)		p value
	%	n	%	n	
Primary					
ACPR	96,9	31	87.9	29	0.003
ETF	0	0	3.0	1	0.259
LCF	0	0	6.1	2	0.520
LPF	3.1	1	3.0	1	0.960
PCR-uncorrected failure rate	3.1	1	12.2	4	0.003
Secondary					
PCT (days)	1.6 ± 0.6		2.4 ± 0.3		0.021
FCT (days)	1.1 ± 0.3		1.5 ± 0.5		0.055

ACPR: adequate clinical and parasitological response; EFT: early treatment failure; LCF: late clinical failure; LPF: late parasitological failure
PCR: polymerase chain reaction; PCT: parasite clearance time; FCT: fever clearance time

Figure 1. Flow chart for the study of plants used in traditional medicine

Figure 2. Interaction of the different members of a multidisciplinary research team, investigating medicinal agents from higher plants

Figure 3. Chemical structures of antiviral 3-methoxyflavones

Figure 4. 4'-7-Dihydroxy-3-methoxy-5,6-dimethylflavone

Figure 5. Antirhinovirus capsid-binding compounds

Figure 6. Coupling product of 3-O-methylquercetin and substituted piperazinyl pyridazines

Figure 7. Indoloquinoline alkaloids isolated from *Cryptolepis sanguinolenta*

Figure 8. Chemical structures of naturally occurring indoloquinolines and synthetic isoneocryptolepines: **(1)** cryptolepine, **(2)** neocryptolepine, **(3)** isocryptolepine, **(4)** isoneocryptolepine, **(5)** N-methyl-isocryptolepinium iodide, **(6)** cryptoquinoline, **(7)** biscryptolepine

Figure 9. N^1,N^1-Diethyl-N^4-(5-methyl-5*H*-indolo[2,3-*b*]quinolin-8-yl)pentane-1,4-diamine

Figure 10. Structures of compounds isolated from the stem bark of *Nauclea pobeguinii*4

Figure 11. Change in parasitaemia during treatment with PR 259 CT1 and AS + AQ.

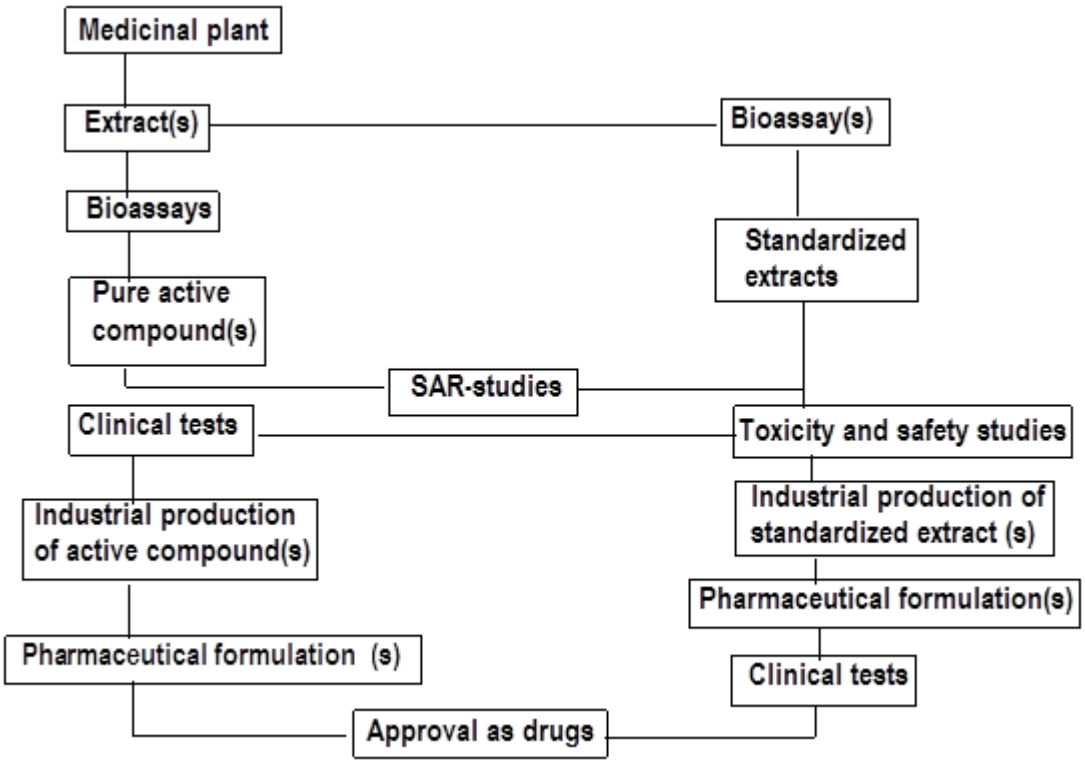


Figure 1. Flow chart for the study of plants used in traditional medicine

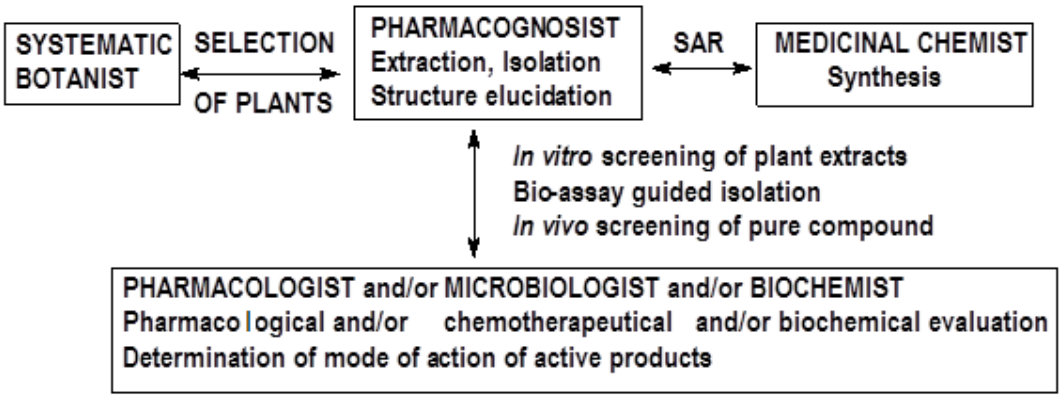
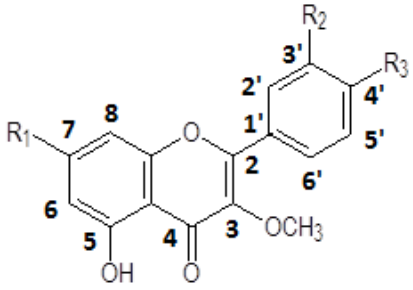


Figure 2. Interaction of the different members of a multidisciplinary research team, investigating medicinal agents from higher plants



R_1	R_2	R_3	Name
OH	H	OH	3-*O*-methylkaempferol
OCH_3	H	OH	3,7-*O*-dimethylkaempferol
OH	H	OCH_3	3,4'-*O*-dimethylkaempferol
OH	OH	OH	3-*O*-methylquercetin
OH	OCH_3	OH	3,3'-*O*-dimethylquercetin
OCH_3	OH	OH	3,7-*O*-dimethylquercetin
OCH_3	OCH_3	OH	3,3',7-*O*-trimethylquercetin

Figure 3. Chemical structures of antiviral 3-methoxyflavones

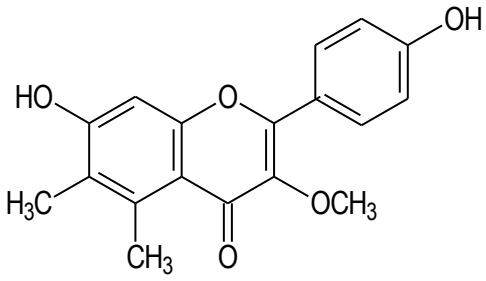


Figure 4. 4’-7-Dihydroxy-3-methoxy-5,6-dimethylflavone

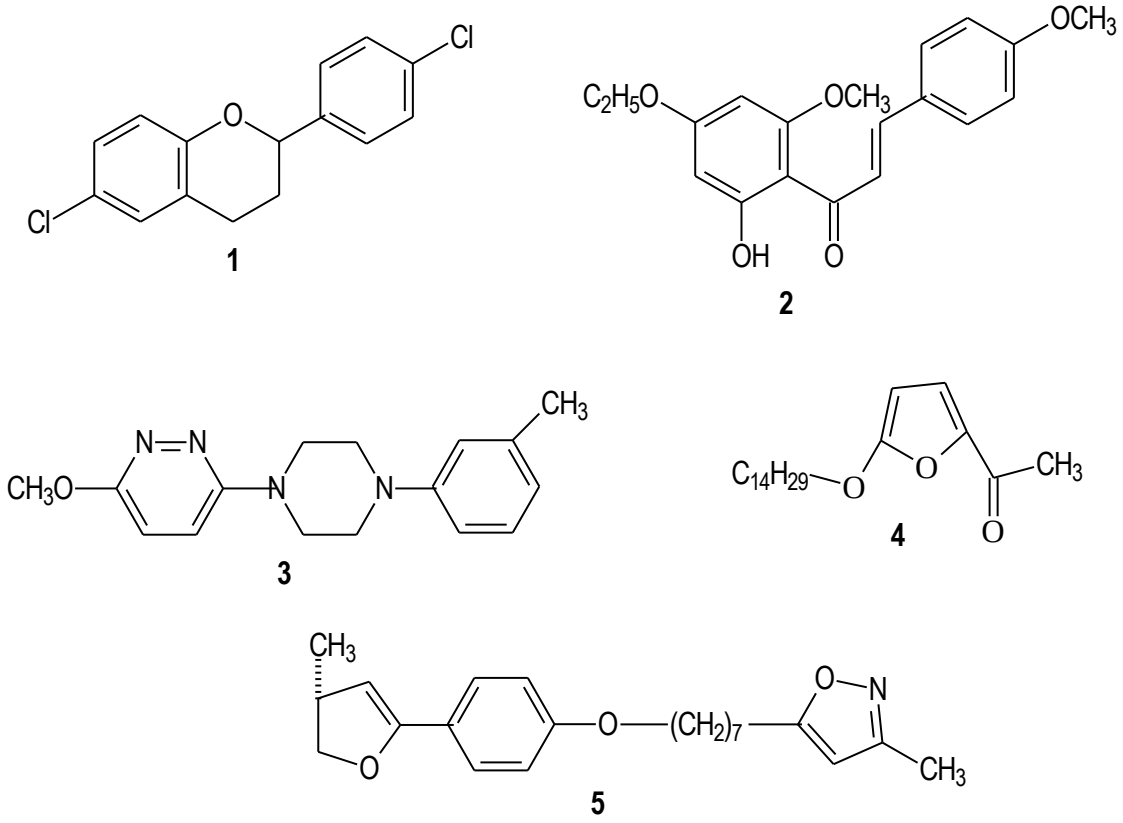


Figure 5. Antirhinovirus capsid-binding compounds

1: 4,’6-dichloroflavan

2: 4’-ethoxy-2’-hydroxy-4,6’-dimethoxychalcone

3: 3-methoxy-6-[4-(3-methyphenyl)-1-piperazinyl]pyridazine

4: 1-(5-tetradecyloxy-2-furanyl)ethanone

5: (*S*)-(-)-5-[7-[4-(4,5-dihydro-4-methyl-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole

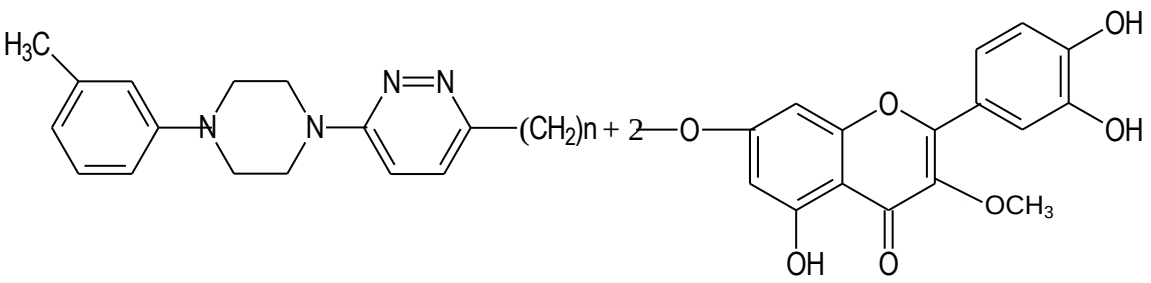
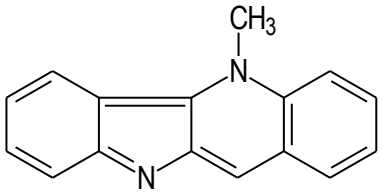


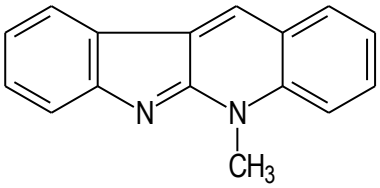
Figure 6. Coupling product of 3-O-methylquercetin and substituted piperazinyl pyridazines



Cryptolepine

(indolo[3,2-b]quinoline

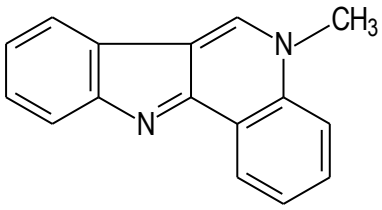
benzo-δ-carboline)



Neocryptolepine

(indolo[2,3-b]quinoline

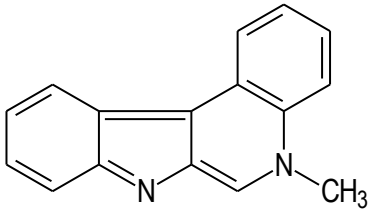
benzo-α-carboline)



Isocryptolepine

(indolo[3,2-c]quinoline

benzo-γ-carboline)



Isoneocryptolepine

(indolo[2,3-c]quinoline

benzo-β-carboline)

Figure 7. Indoloquinoline alkaloids isolated from *Cryptolepis sanguinolenta*

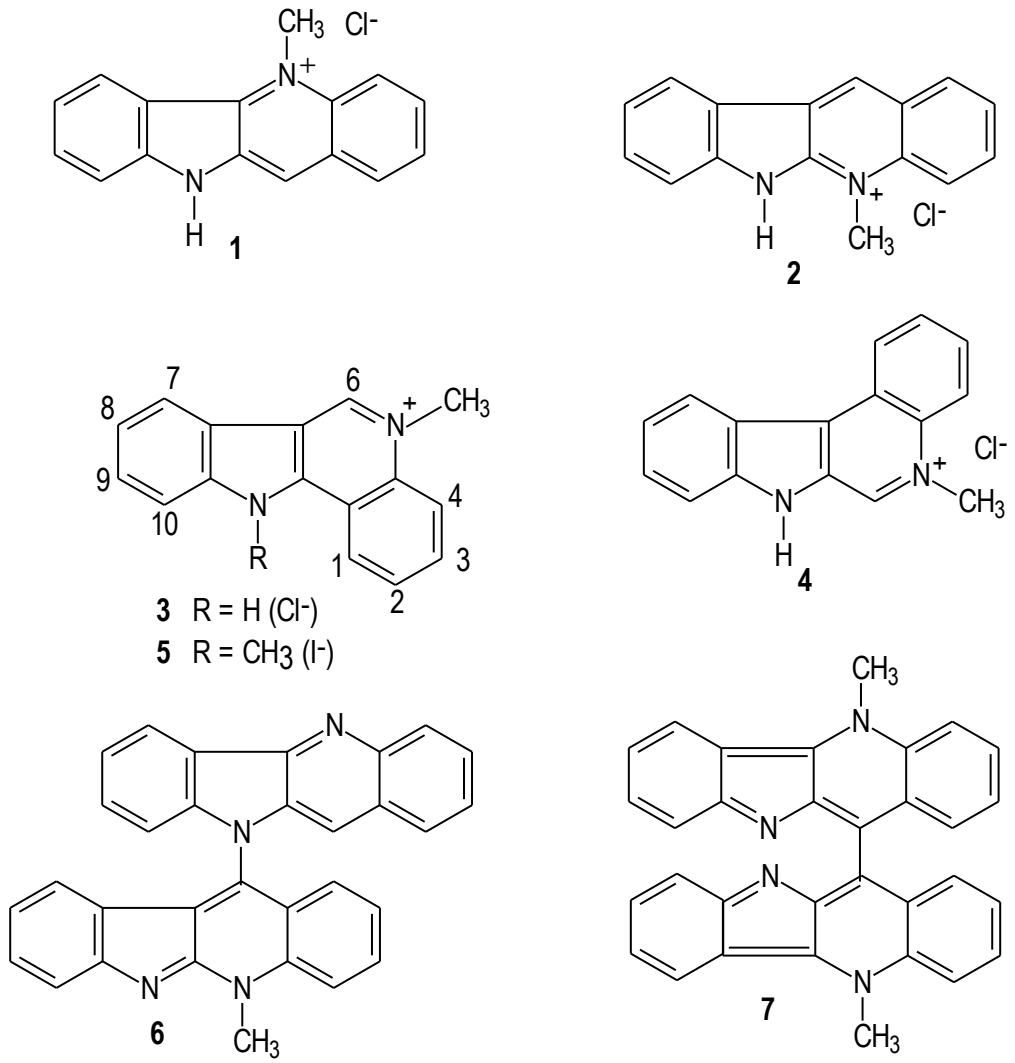


Figure 8. Chemical structures of naturally occurring indoloquinolines and synthetic isoneocryptolepines: **(1)** cryptolepine, **(2)** neocryptolepine, **(3)** isocryptolepine, **(4)** isoneocryptolepine, **(5)** N-methyl-isocryptolepinium iodide, **(6)** cryptoquinoline, **(7)** biscryptolepine

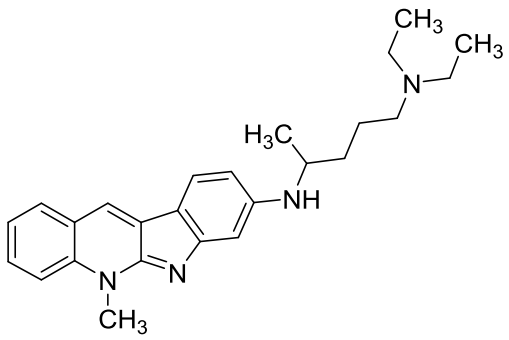
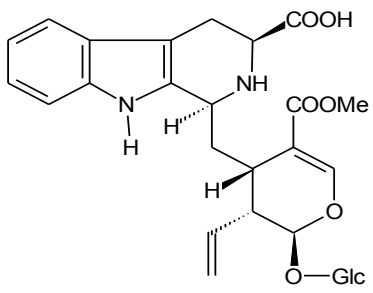
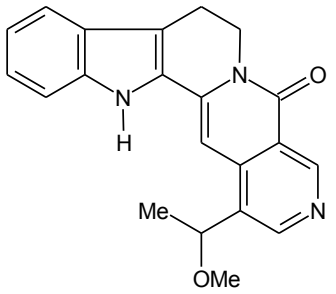


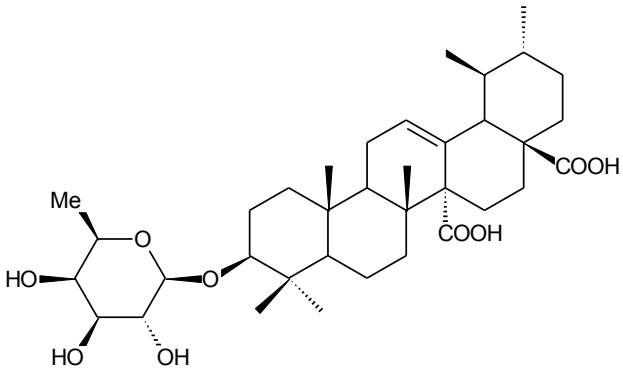
Figure 9. N^1,N^1-Diethyl-N^4-(5-methyl-5*H*-indolo[2,3-*b*]quinolin-8-yl)pentane-1,4-diamine



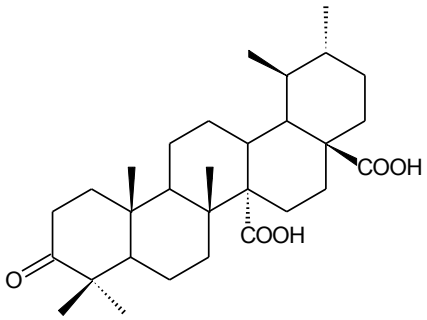
5(*S*)-5-carboxystrictosidine



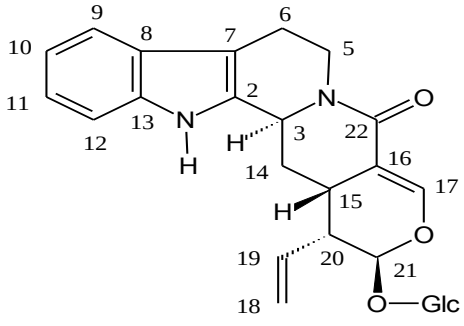
19-*O*-methylangustoline



3-*O*-β-D-fucosyl-quinovic acid



3-keto-quinovic acid



strictosamide

Figure 10. Structures of compounds isolated from the stem bark of *Nauclea pobeguinii*

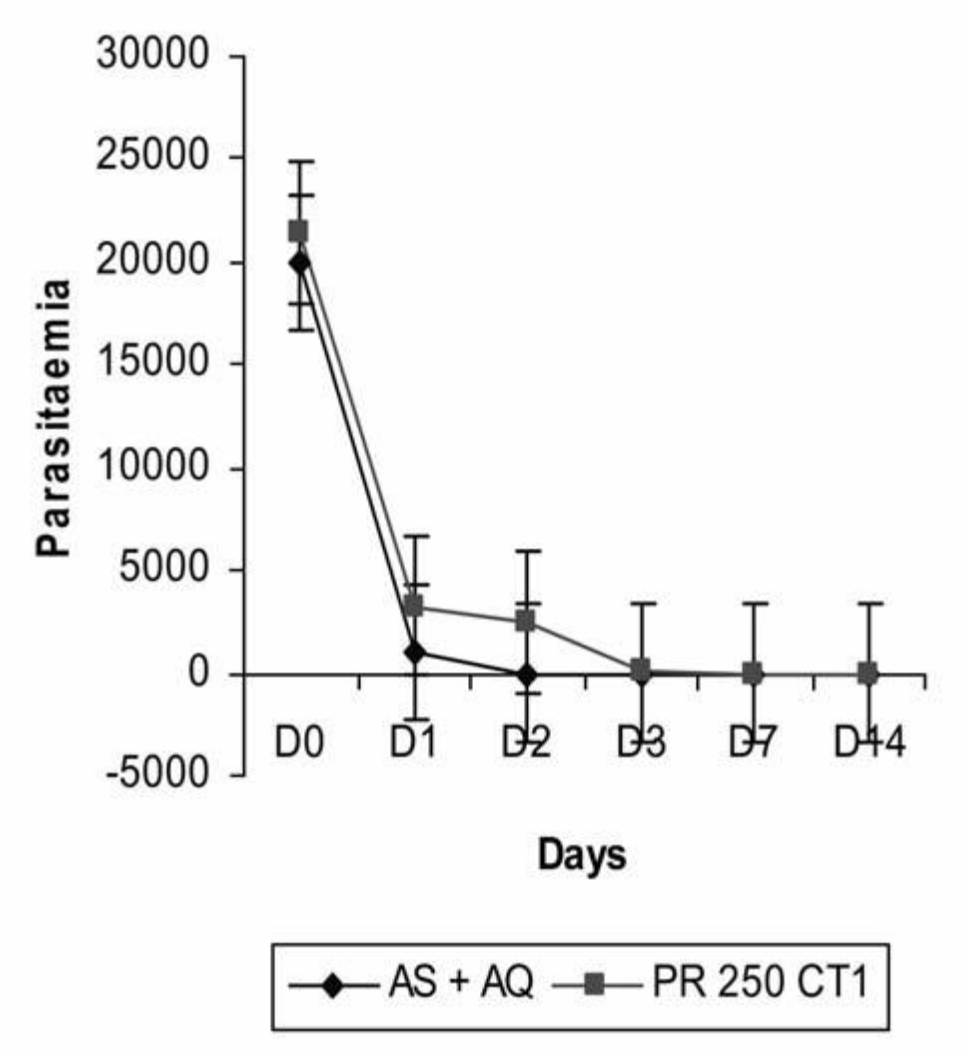


Figure 11. Change in parasitaemia during treatment with PR 259 CT1 and AS + AQ.