DISCOVERY OF NATURAL ANTIPROTOZOALS FROM MEDICINAL PLANTS *SAUSSUREA COSTUS* AND *CARICA PAPAYA*

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
CDC	Centers for Disease Control and Prevention
CFS	Cerebrospinal fluid
DALY	Disability-adjusted life years
DNDi	Drugs for Neglected Disease initiative
ELSD	Evaporative light scattering detector
ESI-MS	Electronspray ionization-mass spectroscopy
FLD	Flourescence detector
HAT	Human African trypanosomiasis
HPLC	High pressure/performance liquid chromatography
HR-MS	High resolution mass spectroscopy
HTS	High throughput screening
IC_{50}	50% growth inhibitory concentration
MMV	Medicines for Malaria Venture
MS	Mass spectroscopy
MS/MS	Tandem mass spectroscopy
NA-DFC	National Agency-Drug and Food Control
NMR	Nuclear magnetic resonance
NP	Natural product
NTD	Neglected tropical disease
OHT	*Obat herbal terstandar* (standardized herbal medicine)
PDA	Photo diode array
ResNetNPND	Research Network Natural Products against Neglected Diseases
SAR	Structure-activity relationship
SI	Selectivity index
TD	Tropical disease
UHPLC	Ultra high pressure/performance liquid chromatography
WHO	World Health Organization

SUMMARY

Medicinal plants have been an important source for the discovery of therapeutic agents for infectious diseases. In order to explore their potential an appropriate selection of plant species is important. In our attempt to find hits targeting antiprotozoal diseases, we utilized an extract library setting, and ethnomedicinal information. A library of 1800 plant and fungal extracts was screened for *in vitro* effects against *Trypanosoma brucei rhodesiense* STIB 900 strain and *Plasmodium falciparum* K1 strain. The ethyl acetate extract of *Saussurea costus* roots, and the methanolic extract of *Carica papaya* leaves were selected for further studies. HPLC-based activity profiling enabled the localization and identification of the active constituents of these plants extracts. Sensitive hyphenated analytical methods such as HPLC-PDA-ESI-TOF-MS and microprobe NMR were used for structure elucidation of the isolated compounds. X-ray crystallography was used in combination with electronic circular dichroism to determine the absolute configuration of selected compounds.

The ethyl acetate extract of *S. costus* roots potently inhibited the growth of *T. b. rhodesiense in vitro*. HPLC-based activity profiling led to the identification of four sesquiterpene lactones. Three structurally related sesquiterpene lactones that originated from different sources were also investigated. All compounds exhibited profound activity against *T. b. rhodesiense* with IC_{50} values between 0.8 – 21.9 μM. Cytotoxicity was tested on rat myoblast L-6 cells, where IC_{50} values of 1.6 to 19.4 μM were observed, and provided selectivity indices (SI) between 0.5 and 6.5. The most active compounds in this study were the germacranolides costunolide, parthenolide, and eupatoriopicrin.

The leaves of the Indonesian ethnomedicinal plant *C. papaya* are a known antimalarial remedy. So far, the active principles have not been investigated from a phytochemical and pharmacological point of view. HPLC-based activity profiling of the methanolic extract from *C. papaya* leaves against *P. falciparum* led to the discovery of five alkaloids and four flavonol glycosides. All compounds exhibited *in vitro* antimalarial activity against *P. falciparum* K1 strain, albeit to varying degrees. Three dimeric alkaloids showed potent activity with IC_{50} values ranging from 0.2 to 1.8 μM, and SI from 24.2 to 107.5. The isolated flavonol glycosides were less active, with IC_{50} values between 13.2 – 16.8 μM, and selectivity indices of more

than 9. Lower activity was observed for the two monomeric alkaloids (IC_{50} ≥ 77 μM). Carpaine (IC_{50} of 0.2 μM; SI of 107.5) was the most interesting compound in this study and was, hence, selected for further evaluation of its *in vivo* pharmacological properties using a 4-day suppressive assay on mice. However, only a reduction of parasitemia by 11.9% was observed. With the aid of X-ray crystallography and ECD calculation, the absolute configuration for carpaine was established as 1*S*,11*R*,13*S*,14*S*,24*R*,26*S*. Carpaine represents a new scaffold for anti-plasmodial drugs. An analysis of carpaine content by means of UPLC-MS/MS was pursued with 28 leaf samples from Indonesia and one from India. The carpaine content varied from 0.02 to 0.31%.

ZUSAMMENFASSUNG

Medizinalpflanzen, die traditionell zur Behandlung von Infektionskrankheiten eingesetzt werden, spielen bei der Entdeckung neuer Wirkstoffe eine grosse Rolle. Für die Identifizierung neuer Leitstrukturen ist zudem die Auswahl geeigneter Pflanzen von entscheidender Bedeutung. Auf der Suche nach antiprotozoal wirkenden Verbindungen wurden sowohl ethnobotanische Informationen als auch die Ergebnisse eines vorausgegangenen Extraktscreenings verwendet. Insgesamt wurden 1800 Pflanzen- und Pilzextrakte auf ihre *in vitro* Aktivtät gegen den *Trypanosoma brucei rhodesiense* STIB 900 Stamm und den *Plasmodium falciparum* K1 Stamm hin untersucht. Der Ethylacetat-Extrakt aus den Wurzeln von *Saussurea costus* und der methanolische Extrakt aus den Blättern von *Carica papaya* wurden für weitere Untersuchungen ausgewählt. Das HPLC-basierte Aktivtätsprofiling ermöglichte die Lokalisierung und Identifizierung der aktiven Extraktkomponenten. Für die Strukturaufklärung der isolierten Verbindungen wurden analytische Methoden wie HPLC-PDA-ESI-TOF-MS und 'Microprobe' NMR verwendet. Die absolute Konfiguration einzelner Verbindungen wurde mittels Röntgenstrukturanalyse und Zirculardichroismus bestimmt.

Der Ethylacetat-Extrakt aus den Wurzeln von *S. costus* hemmte das Wachstum des *T. b. rhodesiense* Stamms nahezu vollständig. Mittels HPLC-basiertem Aktivitätsprofiling konnten vier Sesquiterpenlactone identifiziert werden. Zusätzlich zu den isolierten Reinsubstanzen wurden drei strukturell verwandete Sesquiterpenlactone im *in vitro* Assay getestet. Alle Sesquiterpenlactone zeigten signifikante *in vitro* Aktivität gegen *T. b. rhodesiense* mit IC_{50} Werten zwischen 0.8 and 21.9 μM. Die zytotoxischen IC_{50} Werte wurde mit der Zellinie L6 (Rattenmyoblasten) bestimmt und reichten von 1.6 bis 19.4 μM. Die Selektivitätsindizes der getesteten Substanzen lagen zwischen 0.5 und 6.5. Costunolid, Parthenolid und Eupatoriopicrin waren die aktivsten Sesquiterpenlactone.

Die Blätter von *C. papaya* werden in der indonesischen Volksmedizin gegen Malaria eingesetzt. Die aktiven Inhalststoffe sind jedoch aus phytochemischer und pharmakologischer Sicht bisher wenig erforscht. Mit Hilfe des HPLC-basiertem Aktivitätsprofiling wurden aus dem methanolischen Blattextrakt insgesamt fünf Alkaloide

und vier Flavonolglykoside identifiziert. Die isolierten Substanzen hemmten alle das Wachstum des *P. falciparum* K1 Stammes, wenn auch unterschiedlich stark. Für die drei dimeren Alkaloide lagen die IC_{50} Werte zwischen 0.2 und 1.8 μM und die Selektivitätsindizes zwischen 24.2 und 107.5. Die Flavonolglykoside waren weniger aktiv, die IC_{50} Werte reichten von 13.2 bis 16.8 μM und die Selektivitätsindizes waren grösser als 9. Eine noch geringere *in vitro* Aktivität wurde für die beiden monomeren Alkaloide beobachtet (IC_{50} ≥ 77 μM). Carpain (IC_{50} von 0.2 μM; SI von 107.5) war die aktivste Verbindung und wurde für weitere *in vivo* Untersuchungen ausgewählt. Im Mausmodell reduzierte die Substanz die Parasitämie nach 4-tägiger Behandlung allerdings nur um 11.9%. Mittels Röntgenstrukturanalyse und Zirculardichroismus wurde die absolute Konfiguration von Carpain als 1*S*,11*R*,13*S*,14*S*,24*R*,26*S* ermittelt. Carpain ist auf Grund seiner einzigartigen Molekülstruktur eine interessante Verbindung bei der Suche nach neuen Antimalaria-Wirkstoffen. Des Weiteren wurde mit Hilfe einer UPLC-MS/MS Methode der Carpain-Gehalt in 29 verschiedenen *C. papaya* Blattproben bestimmt. Der Carpain-Gehalt schwankte zwischen 0.02 und 0.31%.

CHAPTER I

AIM OF THE WORK

The therapy for neglected tropical diseases caused by protozoan infections is in an urgent need for the discovery of new therapeutic agents. Current antitrypanosomal drugs for human African trypanosomiasis are mostly old and have been reported to have severe side effects. For treatment of malaria, potent antimalarial drugs are indeed available. However, resistance against these drugs is currently appearing.

Natural sources such as plants continuously supply natural products and drugs derived from natural products for therapy of diseases. The fact that antimalarial drug discovery has successfully relied on natural products is a good reason for exploring medicinal plants for the discovery of new natural products targeting protozoan infections.

The objective of the present work was a phytochemical investigation of medicinal plants, in order to discover bioactive compounds inhibiting *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*. Initially, a screen was conducted on 1800 plant and fungal extracts from an in-house extract library. In this preliminary screening, the extract of *Saussurea costus* was found to be active. A different approach was pursued by following the traditional knowledge on Indonesian medicinal plants. With this, *Carica papaya* was selected for its empirical use and previous reports on *in vivo* activity of the extract.

Subsequently, an HPLC-based activity profiling was applied to track the constituents responsible for the activity within the extracts. Simultaneously, this approach was also used for early identification and dereplication as well as assessment for lead potential of the constituents. Isolation of the active compounds was performed with the aid of diverse chromatographic methods, and the structures were elucidated by means of spectrometric and spectroscopic methods. Additional compounds within the extract with structures related to the active constituents were also isolated to draw preliminary structure/activity relationships. The final aim of this research was to find active compound(s), preferably with new scaffolds, that fulfilled the requirements for progression to *in vivo* screening in a mouse model. Additionally, in the case of papaya leaves, the content of active principle in the sample materials was determined using a validated analytical chromatographic method.

CHAPTER II

INTRODUCTION

1.1 Tropical diseases

All infectious diseases that occur principally in the tropical countries are referred to as 'tropical diseases' (TDs) (Zumla and Ustianowski, 2012). Amongst the WHO list of TDs, 17 diseases are considered as neglected (WHO, 2010b). These diseases impact mostly impoverished populations in remote or isolated areas that are relatively clustered where the victims have low profiles and statuses in public health priorities, and are therefore called as 'neglected tropical diseases' (NTDs). Six types of infectious organisms are the cause of NTDs: protozoan, helminth, viral, bacterial, fungal, and ectoparasites. Protozoan infections cause leishmaniasis, human African trypanosomiasis (sleeping sickness), and human American trypanosomiasis (Chagas disease) (Hotez et al., 2009). Malaria is another protozoan infection ailment that is no longer categorized as NTD because of improved awareness and efforts to combat this disease in the recent years.

CDC estimated that worldwide, there are 149 countries and territories which are affected by at least one NTD. Furthermore, in 56 low-income countries, at least five NTDs were coendemic (Hotez et al., 2009). Approximately 534,000 people worldwide are killed per year because of NTDs with most victims being children (Hotez et al., 2007).

Three parallel approaches have been programmed to eradicate NTDs including vector control, drug treatment, and vaccination. In regard to drug treatment, many of the current drugs are old and have been reported to possess drawbacks in therapy along with rising of resistance, so that there is an urgent need for new safer, effective, and affordable medicines. Since these diseases mostly impact some of the poorest populations in the world and are exclusively transmitted in tropical and sub-tropical countries, they are commercially unappealing to pharmaceutical companies due to low investment return. In the late 1990s, most of pharmaceutical companies stopped their drug research and development for tropical diseases mainly malaria. In concern to this situation, WHO facilitated the creation of the nonprofit public-private partnership organization, 'Medicines for Malaria Venture' (MMV) in 1999 (MMV, 2014). Today, MMV is engaged with 18 founding partners to finance its research programs; amongst them are WHO, Roll Back Malaria, World Bank, Gates Foundation, and government agencies from Switzerland, United Kingdom, Ireland, US and Netherlands. In 2003, another non-profit drug research organization targeting NTDs called

'Drugs for Neglected Disease Initiative' (DNDi) was also formed. The involvement of academic research centers in the recent years has also contributed into this progressing effort. One of them was a joint research of scientists from different countries focusing on drug findings from natural origins named 'Research Network Natural Products against Neglected Diseases' (ResNetNPND) that was established in 2011.

1.1.1 Human African trypanosomiasis

Vector and parasite

Human African trypanosomiasis (HAT), also named as sleeping sickness, is an infectious disease caused by the protozoan parasite *Trypanosoma brucei* (*T.b.*) transmitted to humans by tsetse flies of the genus Glossina. The parasite infects human with two sub-species: *T. b. gambiense* and *T. b. rhodesiense* (Malvy and Chappuis, 2011). *T. b. rhodesiense* causes acute HAT while *T. b. gambiense* causes chronic HAT. *T. b. gambiense* can also infect wild animals in forest areas and domestic animals such as sheep, goats, and pigs (Njiokou et al., 2006, 2010), which indicates the role of animals as reservoir host of the parasite.

Epidemiology

WHO reported a decrease of HAT incident in the past 10 years and left 6743 new cases from African region (Simarro et al., 2013). However, the number of infection cases was estimated to be three times higher (Simarro et al., 2011). In the area of sub-Sahara Africa, HAT is endemic in 36 countries. The most prevalent chronic gambiense HAT is transmitted in 24 countries in Western and Central Africa, whilst the acute rhodesiense HAT is spread in 13 countries in Eastern and Southern Africa.

Disease symptoms and diagnosis

The infection is initiated by transmission of the unicellular trypanosomes via the fly bite during the blood-feeding process. Then the parasites live and multiply extracellularly in the blood and tissue fluids of their human host (Malvy and Chappuis, 2011). HAT occurs in two stages. Stage 1, the haemolymphatic phase, includes non-specific symptoms like headaches and bouts of fever without CNS disorders. Stage 2, the later neurologic phase, occurs when the parasite crosses the blood-brain barrier. In this stage CNS is influenced and causes

serious apparent symptoms like sleep cycle disruptions, paralysis, and progressive mental deterioration. Typical sleep disturbances, where the normal night sleeping time is distorted to daylight and vice versa, are the characteristics of the so-called *sleeping sickness* (Brun et al., 2010). While gambiense sleeping sickness lasts for months to years, the incubation time of acute rhodesiense sleeping sickness is within weeks or months. If untreated the second stage of the disease is lethal.

Simple diagnosis of trypanosomiasis relies on microscopic detection of trypanosomes in the blood, lymph nodes aspirate, and cerebrospinal fluid (CSF). Disease stage determination can only be determined using CFS sample.

Drug treatment and resistance

Due to the absence of vaccines, chemotherapy remains the primary means for control of HAT (Brun et al., 2010). Five available drugs for this ailment are pentamidine, eflornithine, melarsoprol, suramin, and nifurtimox. The first four drugs are delivered intra-venously. All these drugs are employed for gambiense infection. While for rhodesiense infection only two drugs are used: suramin for the blood phase condition and melarsoprol for the neurologic phase condition.

Pentamidine is the drug of choice for treatment of first stage condition. It is given intramuscularly for a week or through intravenous infusion in saline (Brun et al., 2010). This drug has been used for over 60 years without any sign of resistance occurring (Delespaux and de Koning, 2007).

Eflornithine was introduced for the treatment of human gambiense sleeping sickness in 1990s. It is a rather slow acting drug, given via intravenous infusion, and used for melarsoprol-refractory sleeping sickness. The frequent adverse reactions are similar to those produced by cytotoxic drugs (Brun et al., 2010).

Melarsoprol, a prodrug of the active form of melarsen oxide, is administered for late stage conditions on both gambiense and rhodensiense sleeping sickness since 1947. Ineffectiveness has been noted in several highly endemic *T. b. gambiense* infectious foci such as Southern Sudan, Democratic Republic of Congo, Uganda and Angola (Brun et al., 2010; Legros et al., 1999; Balasegaram et al., 2006). The most severe side effects with melarsoprol are

encephalopathies that occur in 5-10% of the treated patients (Blum et al., 2001; Pépin and Milord, 1994).

Suramin has been used since 1920 for only first stage HAT because it does not cross the blood brain barrier (Hawking, 1940). After prolonged use over 80 years, no resistance to suramin has developed (Fairlamb, 2003).

Nifurtimox is the only HAT drug administered orally. This drug was initially known for the treatment of American trypanosomiasis, Chagas disease in the 1960s (Wegner and Rohwedde, 1972). Nifurtimox is well tolerated by patients with melarsoprol-refractory gambiense sleeping sickness (van Nieuwenhove and Declercq, 1989; van Nieuwenhove, 1992).

Drug combination of current trypanocidals has been considered as one option for therapy in view of the absence of new medicines in clinical trials (Keiser et al., 2001). One of the results is the drug combination of nifurtimox-eflornithine for second stage gambiense HAT (Alirol et al., 2013).

Despite the many decades of use of most of the current trypanocides, their mode of action is still limitedly understood. Possible mechanisms proposed include action on multiple targets inside the cell and selective accumulation by the pathogen (Delespaux and de Koning, 2007).

For future elimination of HAT, new antitrypanosomal drugs that are safe, effective, affordable, and preferably with simple or oral administration to treat patients with both stages of the disease are the main goals in drug discovery and development.

1.1.2 Malaria

Vector and parasites

Malaria is a mosquito-borne infectious disease. The vector, Anopheles mosquitoes, injects protozoan plasmodiums into human through their bites. In Nature, there are more than 100 protozoan parasites *Plasmodium sp.* that infect different organisms from human to animals such as birds, reptiles, rodents, primates and other mammals with their individual vectors (Garnham, 1966). Malaria in human is caused by five *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. *P. knowlesi* is found originally in primates

(macaque monkeys). This parasite spread out around South-East Asia and can be somehow transmitted to human. Among these parasites *P. falciparum* is the most severe and deadly. It is also predominant in the endemic areas along with *P. vivax* (Greenwood et al., 2005; Singh et al., 2004).

Epidemiology

According to the latest report (WHO, 2013a), 3.4 billion people are still at risk of malaria. From this total, 1.2 billion of people reside in the high risk malaria areas covering the African region (47%) and South-East Asia (37%). In 2012, there were about 207 million cases of malaria that caused 627 thousand of lethal cases. Victims were mostly African children, which accounted for 77% of the cases. Malaria is preventable and curable. Therefore, with the continuous prevention and control, the mortality rate has successfully slowed down to 45% globally and 49% in the WHO African region since the year of 2000.

Although malaria is almost exclusively found in tropical countries, with the current global climate change and high travelling traffic to and from infected areas, transmission risk to malaria-free areas may occur (Sebisubi and Tan, 2010).

Disease symptoms and diagnosis

Malaria is divided into uncomplicated and severe malaria. Uncomplicated malaria has a typical symptom of recurrent paroxysm that consists of a cold stage onset, followed by high fever stage, and then the sweating stage. Other nonspecific symptoms are flu-like syndrome, headache, myalgia, weakness, vomiting, and jaundice (Warrel, 1993). Uncomplicated malaria can lead to severe malaria where damage of the brain (cerebral malaria) and vital organs occur. If untreated, it can be fatal.

Malaria is conventionally diagnosed by microscopic parasitemia (parasites count) on the patient's peripheral blood smear after staining with Giemsa or other stain solutions (Warhurst and Williams, 1996). Other non-conventional diagnosis methods for parasitemia are also available such as Rapid Diagnostic Test (Murray and Bennet, 2009), PCR analysis (Kawamoto et al., 1996), and serology test (Spencer et al., 1981).

Drug treatment and resistance

Endemism of malaria has been reduced over the last decade. In 2013, only 97 countries were still having on-going malaria transmission (WHO, 2013a). One of the important factors which contributed to this improvement is the availability of affordable, safe, and effective medicines.

The choice of the drug is determined based on the disease severity and the type of the parasite. Current recommended chemotherapy agents are *artemisinin-based combination therapies* (ACTs) for uncomplicated falciparum malaria including arthemether-lumefantrine, artesunate-amodiaquine/mefloquine/sulfadoxine-pyrimethamine, and dihydroartemisinin-piperaquine (WHO, 2013a). Combination therapy is important to slowdown drug resistance (White et al, 1999). For severe falciparum malaria, current first line therapy is parenteral artesunate. Parenteral quinine is the second-line agent. Artemisinin and its derivatives arthemether and artemotil are prescribed as well. When other drugs fail quinidine is the last-line chemotherapy agent (Mondorb et al., 2010; WHO, 2010a).

For the treatment of vivax malaria, WHO´s first recommendation remains chloroquine or chloroquine-primaquine combination, except in some countries where chloroquine resistance is prevalent. ACTs paired with primaquine is in use in the case of chloroquine resistance (WHO, 2010a). Amodiaquine is also an alternative choice (Maguire et al., 2006).

Other malaria parasites, *P. ovale* and *P. malariae* in general are still sensitive to chloroquine. In the case of relapsing ovale malaria, chloroquine-primaquine combination is in use (WHO, 2010a). Further drugs are also incorporated in malaria prophylaxis. Amongst them are the antibiotic doxycycline, mefloquine, atovaquone-proguanil and chloroquine-proguanil (PHE, 2013). Several other sulfonamides, antifolates and antibiotics are also in use for the treatment of malaria (Schlitzer, 2007; WHO, 2010a).

P. falciparum has developed resistance to almost all single use of antimalarial drugs. Resistance to quinine, chloroquine, sulfadoxine-pyrimethamine, and even amodiaquine has been reported from almost all endemic malaria countries between the periods of the late 1950 to 1980s. Treatment failures with mefloquine, then later with artemisinin, have also been found in four South-East Asia countries: Cambodia, Myanmar, Thailand, and Vietnam (WHO, 2013a). Progressing resistance to ACT such as artesunate-mefloquine has also developed in the area of Cambodian-Thai border (Alker et al., 2007). This concern has led

the WHO to establish a strategic guidance, the 'global plan for artemisinin resistance containment' in 2011.

Chloroquine resistant vivax malaria has also progressed in several countries: India, Indonesia, Vietnam, Myanmar, Madagascar, Ethiopia, and Guyana (Dua et al., 1996; Asih et al., 2011; Phan et al., 2002; Barnadas et al., 2008; Guthmann, 2008; Tulu et al., 1996; Phillips et al., 1996). In Indonesia, this drug was also found to be ineffective against *P. malariae* (Maguire et al., 2006).

1.2 Discovery of hits from natural sources

Natural sources including plants, animals, and minerals have been used in human history to promote and maintain health. Historically, several today important medicines such as morphine, quinine, and atropine are originated from ethnomedicinal plants (Hesse, 2002; Smith, 2007). In the modern time, approximately 60% to 70% of the world population is still using traditional medicine (Fabricant and Farnsworth, 2001; WHO, 2000).

Natural products show higher chemical diversity than synthetic compounds. Most of natural products are small molecules. They are commonly known as secondary metabolites and are enzymatically constructed and evolutionary optimized in organisms for defense against predators and environmental challenges (Feher and Schmidt, 2003). Therefore, these molecules are more drug-like in comparison to synthetic compounds (Tan, 2005; Singh and Culberson, 2010).

Nature, predominantly by plants, has continued to be an important source of natural products. They served as the drug substances, leads, and templates for the creation of semisynthetic and synthetic drugs. Over the past 30 years, natural products and natural product derived drugs have contributed to the overall new small molecule drugs by approximately 5.6% and 30%, respectively. Moreover, a high portion of synthetic drugs is still related to natural products, via mimicking or bearing pharmacophores of natural products (Newman and Cragg, 2012). For the treatment of infectious diseases, natural products and their derivatives have played a significant role as antimalarial drugs and antibiotics.

Only parts of the plants have been investigated chemically and for bioactivity. While there is no consensus on the number of world plants that have been studied, a case study for the Swiss flora revealed than from 2677 native species only 55% has been chemically studied and 28% has been tested for bioactivity (Adams et al., 2013). Therefore numerous novel scaffolds remain to be discovered. Moreover, a large number of the reported natural products have not yet been tested for bioactivity or have been assayed only against a limited number of targets.

The success of natural product research depends on several aspects including the plant selection, screening procedures, pharmacological models, and fractionation process (Bourdy et al., 2008).

Plant selection for drug discovery

In natural product research that aims at the finding of new scaffolds, biodiversity of plants is more important than their numbers. Plants from taxonomic groups which have been little investigated or from families known to produce a large diversity of secondary metabolites may be particularly attractive.

Plant species chosen for studies can be selected randomly or systematically. Random plant selection is adapted for phytochemical screening that is not necessarily related to the bioactivity. Blind bioactivity testing, without prior knowledge of the plants, leads to a trial and error process with higher chance of failing and the risk of missing the actual activity of a plant species (Zhang, 2005). This approach was frequently applied in the past. Systematic selection of plant species is preferred in the current drug discovery from Nature. Two approaches pursued are chemotaxonomy- and ethnomedicine-based plant selection (Potterat and Hamburger, 2008). Species with close taxonomic relationship often contain rather similar phytochemicals.

The natural materials employed as drugs following the custom of specific cultures are called as traditional medicines. The knowledge of traditional medical practice including the type of plants used is usually passed through generations from their ancestors. Prolonged use of ethnomedicinal plant knowledge for ages or even centuries, in a way, is a proof of efficacy, well tolerance, and safety. This gives a hint of the presence of bioactive molecule(s) in the plants.

Screening procedures

In the past, natural products derived drug discovery employed classical bioassay-guided fractionation techniques. This process is known to be laborious and time consuming (Potterat and Hamburger, 2008). The development of HPLC and UHPLC coupled to highly sensitive detection techniques (MS, PDA-MS, MS/MS, MS-NMR, ELSD, and FLD) has made

early compounds identification and dereplication possible (Potterat and Hamburger, 2013) that in turn greatly improved time efficiency and straightened the isolation process.

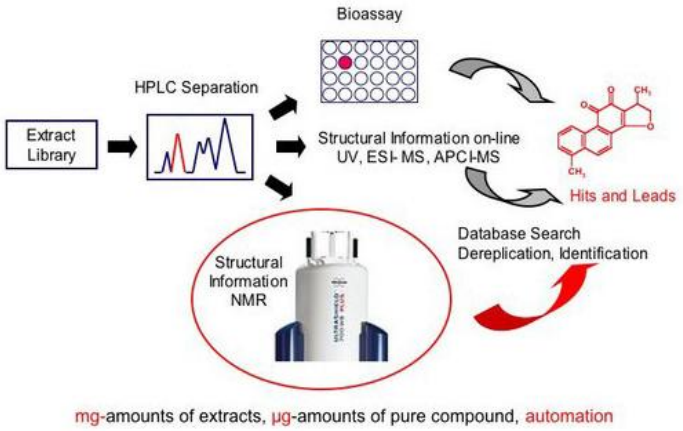

Figure 1: Miniaturized HPLC-based activity profiling approach. (Courtesy of M. Hamburger)

Hyphenated techniques have become the standard method for compound early dereplication and isolation from extracts in modern drug discovery. In HPLC-based activity profiling (Potterat and Hamburger, 2013), these methods are combined with time-based microfractionation of extracts. The fractions are tested for bioactivity. In this way, the activity can directly be correlated to the compound(s) in the extracts.

Bioassay of natural products

Testing of bioactivity on natural product samples is done on extracts, fractions, or compounds. Three generally used types of bioassays are *in vivo* assays on animal models, cell based *in vitro* assays, and biomolecular target based *in vitro* assays. To avoid unspecific results, the type of assay needs to be adjusted according to the tested material. For example, extracts containing tanins are known to interfere with enzymatic assays. Saponins have a tendency to disrupt and produce misleading results in cellular assays (Potterat and Hamburger 2006). Moreover, discrepancies are often observed between *in vitro* and *in vivo* results.

Bioactivity testing on the molecular level has become an attractive approach in the recent years. The completion of genomic sequence of *Plasmodium falciparum* (Gardner et al., 2002) and *Trypanosoma brucei* parasites (Berriman et al., 2005) provides a comprehensive understanding of the parasites biology. This fundamental knowledge can be transferred into applied research for discovery of effective drugs and vaccines (Duraisingh et al., 2006). In the area of antimalarial research, enzyme targets such as *pf*FabI, *pf*FabG, *pf*FabZ have been

incorporated for lead finding from plants and marine organisms (Jensen et al., 2012; Karioti et al., 2008; Kaya et al., 2011; Tasdemir et al., 2010).

In the recent years, computational methods have also been incorporated for prediction of natural product bioactivity at the molecular level. Two different approaches are used; the ligand-based pharmacophore model for structure-activity relationship (SAR) study of natural products, and the structure-based pharmacophore model that is useful for docking studies (Rollinger et al., 2006). This method has also been used for *in silico* screening of natural products for their antitrypanosomal and antileishmanial activities (Schmidt et al., 2014).

1.2.1 Antiprotozoal compounds discovery using extract libraries

A large number of medicinal plants have been reported for antiprotozoal activity from many parts of the world. With this large number of potentially active plant species, there is need for efficient use of time and resources. Working with a library enables a considerable simplification of sample and data handling (Potterat and Hamburger, 2014). Moreover, using standardized procedures for extraction of all plant materials and redissolution of extracts for bioassays ensure the comparability of the activity results. Therefore, the most promising extracts among the extracts stored in the library can be identified.

An extract is a complex mixture of compounds; thereby screening extracts in principle increases the number of tested compounds. Therefore, an increasing discovery rate of hits can be expected. There are, however, several issues which have been recognized when incorporating extract samples into high-throughput screening such as a low content of active compounds in the extract that are below the screening thresholds and the presence of major metabolites covering the active constituents (Bugni et al., 2008). In such cases, fractionation of the extracts prior screening can be an option.

Initially, extract libraries have been established in pharmaceutical and biotech companies. In the recent years, this setting has been also adopted in several academic research groups (Bugni et al., 2008; Chichioco-Hernandez and Villasenor, 2009; McCloud, 2010; Potterat and Hamburger, 2013). The research group of Hamburger and coworkers from the University of Basel has successfully implemented this setting in its search for new antiprotozoal drugs.

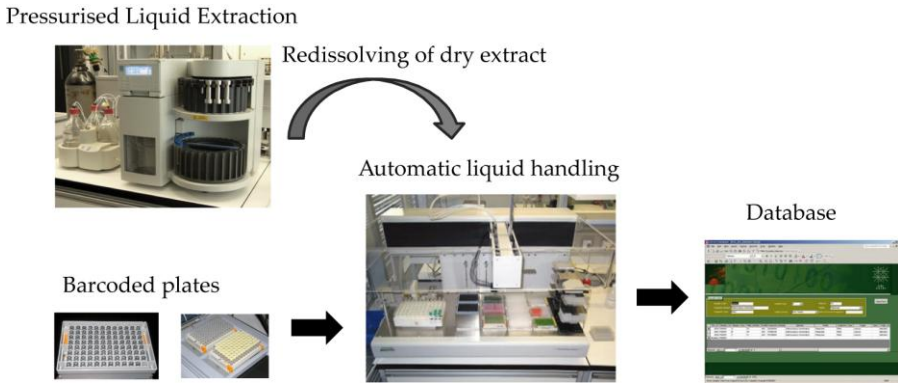

Figure 2: The establishment of an extract library. Automatic pressurized liquid extraction is implemented for sample extraction. Extracts are dried and redissolved in DMSO. Extract solutions are transferred into racked microtubes in 96-well format using a liquid handler. Each tube is barcoded and linked to the extract information stored in a customized database. (Figure courtesy of M. Hamburger, with modification)

Screening of the extract library has led to the identification of a number of hits. Further fractionation of these extracts have resulted in the discovery of bioactive natural products belonging to various compound classes such as lanostanes (Adams et al., 2010), protostane triterpenoids (Adams et al., 2011a), sesquiterpene lactones (Zimmermann et al., 2012; Mokoka et al., 2013), pyrethrin terpenoids (Hata et al., 2011), tanshinone diterpenoids (Ślusarczyk, 2011), abietane diterpenes (Mokoka et al., 2014), isoflavan quinones (Hata et al., 2013; Hata et al., 2014a), and phenanthrenones (Hata et al., 2014b).

Two amongst the isolated compounds have been tested in murine models. A new phenanthrenone scaffold from the stems of *Drypetes gerrardii* (Putranjivaceae) was tested for antiplasmodial activity (Hata-Uribe et al., 2014b), and a sesquiterpene lactone, cynaropicrin, from the aerial parts of *Centaurea salmantica* L. (Asteraceae) was tested for antitrypanosomal activity (Zimmermann et al., 2012). The latter was the first natural product with trypanocidal activity *in vivo*.

1.2.2 Ethnopharmacology-based discovery of antiprotozoal compounds

An analysis in 2001 reported that many of the plant-derived drugs in the market present indications identical to those of their respective ethnomedicinal plants (Fabricant and Farnsworth, 2001). This indirectly shows that ethnomedicinal plants are potential sources of

bioactive molecules for drug finding and development. More hits are likely to be found from traditional medicinal plants (Farnsworth and Kaas, 1981). For example, in the finding of GABA-A receptor modulators, incorporating ethnomedicinal samples into the extract library for screening led to higher discovery of hits (Zaugg, 2011).

Traditional medicines have been reported from numerous countries with diverse therapeutic indications. However, the majority of these traditional medicines have not been explored (Cordell and Colvard, 2012). A study by Adams et al. (2011b) on European Renaissance's antimalarial remedies shows that a large part of the cited plants has never been studied for the reported indication.

In the area of antiprotozoal drugs, two most successful plant-derived antimalarial drugs are quinine and artemisinin. Quinine was firstly isolated from the bark of *Cinchona* spp. (Rubiaceae). The Incas in Peru traditionally used these plants to treat fever. Artemisinin was firstly isolated from the leaves of *Artemisia annua* (Asteraceae). This plant was a Chinese folk medicine for a chill and fever onset corresponding to symptoms of malaria (Wright, 2005). Quinine and artemisinin have become the prototypes for the development of other antimalarial drugs with better pharmacokinetic properties.

Antimalarial drugs belonging to 4-aminoquinolines (e.g. chloroquine, amodiaquine, mefloquine) and 8-aminoquinolines (e.g. primaquine) are synthetic drugs structurally inspired by the alkaloid quinine. The sesquiterpene lactone artemisinin bearing a unique endoperoxide moiety has been the starting point for the development of the semi-synthetic derivatives dihydroartemisinin, artemether, arteether, artemotil and artesunate.

Several bioactive leads derived from ethnomedicinal plants targeting protozoan neglected diseases have been reported in literature. Structurally, they belong to several different compound classes such alkaloids, terpenes, flavonoids, quinones, lignans, xanthones and others (Schmidt et al., 2012a, 2012b; Maas et al., 2011; Ramalhete et al., 2010; Batista et al., 2009). Several compounds should be pointed out for their potent activities (IC_{50} below 1 μM) reported in the last five years. They include two abietane diterpenoids, Δ9-ferruginol and ferruginol from the Iranian medicinal plant *Salvia sahendica* (Lamiaceae) (Ebrahimi et al., 2013); the triterpenoid perovskone B from the Iranian medicinal plant *Salvia hydrangea* (Lamiaceae) (Farimani et al., 2011); ellagic acid from the Nigerian medicinal plant

Chrozophora senegalensis (Euphorbiaceae) (Garcia-Alvarez et al., 2013); quassin and neo-quassin from *Quassia amara* (Simaroubaceae) (Mishra et al., 2010); isocryptolepine from the West African plant *Cryptolepis sanguinolenta* (Apocynaceae) (Whittell et al., 2011); and one isothiocyanate glycoside from the ethnomedicinal plant *Moringa peregrina* (Moringaceae) (Ayyari et al., 2014).

A less number of compounds have also exhibited *in vivo* activity in animal models in the same period of last five years: the isothiocyanate glycoside isolated from *M. peregrina* (Moringaceae) displayed potent antitrypanosomal activity *in vitro*. In a further test in HAT mice model, a temporary 95% reduction of parasitemia was observed before the occurring of relapse on day 10 (Ayyari et al., 2014). Gomphostenin and acetyl-gomphostenin from the Indian plant *Gomphostemma niveum* (Lamiaceae) showed reduction of parasitemia by 81% and 92%, respectively, with survival days of more than 20 (Sathe et al., 2010).

1.3 Indonesia: traditional medicines and malaria

1.3.1 Traditional medicines

Indonesia is a tropical archipelagic country with more than 17 thousand islands, and inhabited by 38 thousands species (Newman et al., 1999). It is estimated that 10% of world's known plant species exist in Indonesia (Wells at al., 1999). The numbers of reported drug plants vary from less than 1900 to more than 2500 (Anonymous, 1995; Zuhud et al., 2001). Only a small proportion of the total therapeutic plants have been incorporated into the databases created by some institutions such as IptekNet with 262 plants and the faculty of pharmacy-University of Airlangga with 196 plants.

Until present, the tradition of utilizing herbal drugs by Indonesians is well implemented on a daily basis. More than 59% of the population is known to use *jamu*, the general term for traditional herbal drugs (Kemenkes RI, 2010). The market share of traditional medicines in national drug consumption has grown to 15% in 2014 from only 1-1.5% back in the 1970s (Sutianto, 2014), and the market growth was reported between 20-30% annually.

History

Figure 3: Relief on Borobudur temple about the tradition of drinking *jamu*. (http://bhumihusadacilacap.blogspot.ch)

The traditional medical system in Indonesia has been semi-documented since early ages in the form of relief on temples, steles, and palm-leaf manuscripts. Only a small part of these ancient manuscripts has been translated and studied. These manuscripts revealed the type of diseases, medicinal plants, modes of preparation, and ways of administration of these medicines (Nawaningrum et al., 2004). During the Dutch colonization, books containing Indonesian medical knowledge were published. The medical information based on Javanese traditions was collected by Horsfield (1813, 1816), Kloppenburg-Versteegh (1907), and Heyne (1917), Moluccan traditions by Bontius (1658) and Rumphius (1747), and Balinese traditions by Weck (1937).

Classification

The National Agency of Drug and Food Control (NA-DFC) of Indonesia defines a traditional medicine as a specimen or remedy either from plant, animal, mineral origin, galenic preparation, or a mixture of these that have been used traditionally for treatment. Therefore, it can be used by the society according to its empirical practice. According to the degree of scientific evidence on efficacy and safety, the NA-DFC divides traditional medicines into three categories: *jamu*, *obat herbal terstandar* (standardized herbal medicine), and *fitofarmaka* (phytomedicine). A basic requirement for all forms of traditional medicines is the existence of good traditional medicine manufacturing practices for their production.

Figure 4: Logo of herbal medicines.

The use of *jamu* is simply based on the traditional knowledge, and its efficacy is confirmed based on empirical evidence. *Jamu* exists in two forms, namely the traditional preparations, and modern dosage forms. Traditional *jamu* is usually a simple decoction of fresh material(s). It is typically sold by street vendors. Modern *jamu* is marketed in dosage forms similar to pharmaceutical products in general, such as powders, pills, capsules, tablets and solutions. Traditional *jamu* is used not only for internal but also for external applications. In modern *jamu*, the ingredients are either powders of simplicia or extracts, in which some are chemically standardized. Safety is guaranteed to the level of no microbial contamination detected. There are over 19 thousand of *jamu* products registered in NA-FDC (Permanasari, 2012).

For *obat herbal terstandar* (*OHT*), the efficacy and safety are preclinically proven. The active ingredients of *OHT* are standardized extracts. There are 38 *OHT* products registered in NA-DFC.

The efficacy and safety of *fitofarmaka* has to be supported by clinical evidence. The active ingredients of *fitofarmaka* are standardized extracts. There are six *fitofarmaka* products registered in NA-FDC up to 2011 (Candra, 2012). *Fitofarmaka* products are considered equivalent to chemical drugs. They can be prescribed by health professionals and are reimbursed by health insurance.

Challenges in the development of herbal drugs

In view of the country's richness in natural resources and traditional drug knowledge, as well as the market potential, it is clear that the field of current modern herbal medicines is severely underdeveloped.

There are several issues that contribute to this unsatisfactory situation. A first reason was the lack of scientific research facilities and funding. Secondly, for earlier studies, the results were frequently kept within the research organizations or academic institutions. Only a small number of results was published in national printed journals as well as international journals, which are often inaccessible online, and therefore not available to the public. Third, a national priority in research on medicinal plants for drug discovery and development was lacking and, finally, research networks of scientists from different institutions were underdeveloped.

In the meantime the government has initiated some adjustments in the academic sector and in national health programs. In the early 1990s, an inventory project on traditional medicinal knowledge in Indonesia was launched. One of the results was the creation of inventory books containing ethnomedicinal information arranged according to provinces. The information came from provinces in Kalimantan (Aziddin et al., 1990; Mudiyono, 1991), Sumatera (Sirat et al., 1990; Ja´far et al., 1990), Bali and Java (Reksodihardjo et al., 1991; Swarsi et al., 1990), and from eastern Indonesian provinces like North Sulawesi (Sarajar et al., 1994) and Maluku (Manuputty, 1990).

A more practical approach was set through a short-term national program released by the government in 2011, for the prioritization of 15 medicinal plants to be exhaustively studied in order to deliver new *OHT* and *fitofarmaka* products. Also, several programs were initiated to increase the extend and quality of medicinal plant cultivation. Among the prioritized medicinal plants were *Andrographis paniculata*, *Psidium guajava*, *Guazuma ulmifolia*, *Piper retrofractum*, *Curcuma domestica*, *Curcuma xanthorrhiza*, *Zingiber officinale*, *Morinda citrifolia*, *Eugenia polyantha*, and *Kaempferia galanga*.

In the academic sector, an indirect program was established to improve the accessibility of research results. Since 2012, scientific publication in journals was set as a requirement for university graduates starting from the Bachelor level.

1.3.2 Malaria and ethnomedicines for malaria

Malaria prevalence

The most prevalent tropical disease in Indonesia, from the first identification in the period of Dutch colonization until the present time, was and still is malaria (Elyazar et al., 2011). However, the continuous eradication efforts have successfully controlled the incidence of this life-threatening disease (Feachem, 2010). According to the WHO last report, in 2012 there were approximately 480 thousand cases of malaria in Indonesia with around 400 cases of death (WHO, 2013a). From a total population of around 240 million people, around 18% are still living in areas of high transmission, 45% in areas of low transmission, and the rest in malaria-free areas. The four human malaria parasites exist in Indonesia, of which *P. falciparum* is the most prevalent. In addition, Knowlesi malaria has been reported in Kalimantan (Berens-Riha et al., 2009).

Malaria drugs

Current first line antimalarial agents are artemisinin combination therapies: artesunate-amodiaquine, artemether-lumefanrtine, and dihydroartemisinin-piperaquine. The combination of dihydroartemisinin-piperaquine is used in the areas where resistance to choloroquine and amodiaquine is prevalent (Harijanto, 2010; Sutanto et al., 2012). Due to the resistance to artemisinin and its combination therapies reported from neighbouring countries, a similar situation is suspected for Indonesia.

Herbal and natural products research from Indonesian medicinal plants

A relatively large number of ethnomedicinal plants with antimalarial properties have been reported in various surveys. Pieces of information were also retrieved from old records. The compiled information usually covered specific survey areas, such as in Kalimantan (Leaman et al., 1995), South-East Sulawesi (Rahayu et al., 2006), and North Sulawesi (Moningka, 1995). A majority of the reports present qualitative data without indicating the number of plant species studied on a scientific level. An analysis on antimalarial plants from Indonesian Papua reported that only three species have been tested for bioactivity from 32 plants in the list (Julianti et al., 2010). Therefore, there is a great opportunity to find new bioactive compounds and scaffolds from the unstudied species.

Most Indonesian studies focused on *in vitro* and *in vivo* testing of extracts and fractions. *In vitro* antiplasmodial activity of isolated compounds was not always tested, and *in vivo* experimental results were rare. Most studies were terminated when the suspected active constituents (usually the major compounds) were chemically characterized from the most active extracts or fractions. Moreover, the bioactive constituents from these Indonesian plants were frequently found to have been reported in previous studies from countries that share similar traditional applications.

Several popular Indonesian medicinal plants with interesting bioactivity against malaria are discussed herein. For plant extracts, only those with a maximum IC_{50} of 10 μg/mL *in vitro* against *P. falciparum* were included. Compounds with *in vitro* activity below 1 μM or 5 μg/mL were considered. Some of the active plants cited from rather old publications had a reported activity as percentage of more than 60% (*in vivo*) or 80% (*in vitro*) instead of concentration.

Carica papaya. The polar alkaloid containing extract of *Carica papaya* leaves displayed *in vivo* activity (Murdiani, 2000; Subeki, 2008). An ethanolic extract was also found to be active *in vitro* (Rehena, 2009).

Cassia siamea. The chloroform-soluble fraction of *Cassia siamea* leaves displayed excellent *in vitro* activity (Ekasari et al., 2004). Further analysis of this extract resulted in the isolation of five aromatic alkaloids with cassiarin A being the most active constituent (Morita et al., 2007; Oshimi et al., 2009). The ethanolic extract of the leaves also demonstrated *in vivo* activity (Ekasari and Widyawaruyanti, 2003).

Alstonia scholaris. The methanolic extract and chloroform-soluble fraction (containing alkaloids) of *Alstonia scholaris* bark showed potent *in vitro* activity (Iwo, 2009; Keawpradub et al., 1999). However, the alkaloid echitamine which was isolated from this plant showed no activity *in vitro* (Wright et al., 1993). An *in vivo* study with *P. berghei* indicated that the petroleum ether and methanolic extracts of the bark possessed activity in a dose dependent manner (Gandhi and Vinayak, 1990).

Andrographis paniculata. An ethanolic extract of the stems of *Andrographis paniculata* (Zein et al., 2013) and a methanolic extract of the whole plant (Rahman et al., 1999) were shown to

have potent *in vitro* activity. From the roots, four xanthones were isolated, whereby 1,2-dihydroxy-6-8-dimethoxy-xanthone was the most active constituent *in vitro*. The compound led also to a 62% reduction of parasitaemia in the mouse model when tested at 30 μg/g BW (Dua et al., 2004).

Brucea javanica. Water extracts of the bark, fruits and leaves of *Brucea javanica* displayed high *in vitro* activity (Murningsih et al., 2005). The chloroform extracts of fruits, leaves, roots, and stems also showed pronounced activity in an animal model (Phillipson and O'Neill, 1986).

Eurycoma longifolia. The quassinoid eurycomanone isolated from a methanolic extract of the roots of *Eurycoma longifolia* displayed excellent activity (Kardono et al., 1991). A further study on this plant revealed another two bioactive constituents namely, 14,15B-dihydroxyklaineanone and eurycomanol, against the *P. falciparum* strain D10 (Chan et al., 2004).

Lansium domesticum. A polar extract of *Lansium domesticum* bark significantly reduced parasitemia to less than 5% (Subeki, 2008). The triterpenoid lansiolide showed antimalarial activity both *in vitro* and *in vivo* (Omar et al., 2003). The seeds were also shown to contain *in vitro* active antiplasmodial compounds, such as domesticulides B and C, methyl 6-acetoxyangolensate, and azadiradione (Saewan et al., 2006).

Acanthostrongylophora sp. In a study on Indonesian marine species, the sponge *Acanthostrongylophora sp*. revealed potent activity against *P. falciparum*, and three active manzamine-type alkaloids were identified (Rao et al., 2004).

Other plants. Several extracts worth mentioning for their pronounced activity in the *P. berghei* mouse model include extracts of *Tinospora tuberculata* stems, *Melastoma malabathricum* leaves, *Arcangelisia flava* stems, *Michelia campaka* bark, and *Imprata cylindrica* rhizomes (Subeki, 2008). Ethyl acetate and butanol-soluble fractions from *Erythrina variegata* showed activity against *P. berghei* (Muhtadi and Haryoto, 2005). Water extracts, with high *in vitro* activity against *P. falciparum* were obtained from *Achillea millefolium*, *Baeckea frutenscens* leaves, *Curcuma xanthorrhiza* rhizomes, *Strychnos lucida* wood, and *Swietenia macrophylla* seeds (Murningsih et al., 2005).

A rather different approach to explore herbal drugs activity was followed in studies with a combination of standard drugs and herbal extracts. A combination of the polar extract of *Lumbricus rubellus* and the commercially available drug chloroquine was administered to mice infected with *P. berghei* and showed increased activity (Wulandari, 2010). Combinations of *Andrographis paniculata* extract with either chloroquine or artemisinin were tested *in vitro.* Higher activity of the combinations was observed in comparison to the extract. However, it was still below the activity of the individual drugs (Zein et al., 2013). Interesting results were obtained with the combination of *Eurycoma longifolia* roots extract with artemisinin. When tested on *Plasmodium yoelli*-infected mice the combination showed higher activity in comparison to artemisinin alone (Mohd Ridzuan et al., 2007).

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CHAPTER III

ANTITRYPANOSOMAL SESQUITERPENE LACTONES

FROM *SAUSSUREA COSTUS*

Antitrypanosomal sesquiterpene lactones from *Saussurea costus*

Tasqiah Julianti, Yoshie Hata, Stefanie Zimmermann, Marcel Kaiser, Matthias Hamburger, Michael Adams. *Fitoterapia* **2011**, *82*, 955-959.

Four sesquiterpene lactones were isolated from the ethyl acetate extract of *Saussurea costus* roots. Their activity was detected with the aid of HPLC-based activity profiling and the structures were established on the basis of high resolution mass spectrometry and NMR spectroscopy. Two sesquiterpene lactones, costunolide and dehydrocostuslactone were the active constituents in *Saussurea costus* along with two other compounds from different sources, parthenolide and eupatoriopicrin against *Trypanosome brucei Rhodesiense in vitro*. In general, higher activity was displayed by three germacranolides: costunolide, parthenolide, and eupatoriopicrin, in comparison to two other compound classes, guaianolides and eudesmanolides.

Extract preparation, HPLC microfractionation, recording and data interpretation for HPLC-based activity profiling, isolation of compounds (except for compounds 5-7), recording and data interpretation for compound´s structure elucidation with spectroscopy methods (HPLC-PDA-ESI-TOF-MS and NMR) (except for compounds 5-7), as well as draft writing and figure preparation for the manuscript are my contributions for this publication.

Tasqiah Julianti

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Antitrypanosomal sesquiterpene lactones from *Saussurea costus*

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ABSTRACT

In the course of a larger screen of 1800 plant and fungal extracts, the ethyl acetate extract of *Saussurea costus* roots potently inhibited the growth of *Trypanosoma brucei rhodesiense*. Subsequent HPLC based activity profiling led to the identification of the sesquiterpene lactones arbusculin B (**1**), α-cyclocostunolide (**2**), costunolide (**3**), and dehydrocostuslactone (**4**). They were tested for in vitro antitrypanosomal activities and cytotoxicity alongside the structurally related sesquiterpene lactones parthenolide (**5**), zaluzanin D (**6**), and eupatoriopicrin (**7**), and had IC_{50}s between 0.8 and 22 μM. Cytotoxic IC_{50}s were from 1.6 to 19 μM, and selectivity indices from 0.5 to 6.5.

1. Introduction

The fragrant roots of *Saussurea costus* (Falc.) Lipschitz (Asteraceae), synonym: *Saussurea lappa* C.B. Clarke, have been used for thousands of years as medicines, incenses and ointments by many cultures. In India they are called Kur or Kushtha, and in China *Yún mù xiāng* (云木香). In the Ayurveda, Siddha, and Unani medicinal systems *S. costus* roots are used alone or in combination with other drugs to treat asthma, cholera, chronic skin diseases, rheumatism, cough and cold, quartan malaria, leprosy, persistent hiccups, rheumatism, stomach-ache, toothache, and typhoid fever [1,2].

A broad spectrum of biological activities such as anti-inflammatory, anticancer, immunomodulatory, CNS depressant, and antimicrobial properties have been reported for *S. costus* extracts [3–5]. Activities have commonly been related to the presence of sesquiterpene lactones. Furthermore, tannins, steroids, alkaloids, glycosides, terpenoids, flavonoids, peptides, and organic acids have been reported from this plant [3,4].

Our interest in this plant was raised when we performed an antiprotozoal screen of 1800 plant and fungal extracts for effects against the parasites *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Plasmodium falciparum* and *Leishmania donovani*, the causal agents of human African trypanosomiasis, Chagas disease, malaria, and leishmaniasis, respectively [6–8]. Amongst the most potent extracts in this screen was an ethyl acetate extract of *S. costus* roots which inhibited *T. b. rhodesiense* by 96% at a test concentration of 4.8 μg/ml.

HPLC based activity profiling was used to identify the active constituents in the extract. In this approach sub-milligramme amounts of extract are separated by analytical scale HPLC and automatically fractionated into 96 well plates. The microfractions obtained are submitted to the bioassay, and the resulting activity profile can be overlaid with the HPLC trace to correlate peaks of activity with peaks in the HPLC chromatogram. On-line spectroscopic data (UV–Vis and MS) collected during separation, combined with database

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searches provide structural information on the active principles [6–8].

We here report on the identification and isolation of antitrypanosomal compounds from *S. costus*, and also on the comparative testing of some related sesquiterpene lactones for antiprotozoal and cytotoxic activities. This study was part of a larger screen for new antiprotozoal leads using HPLC based activity profiling which has led to the identification of numerous active compounds [8–11].

2. Materials and methods

2.1. General experimental procedures

Analytical grade solvents for extraction and HPLC-grade eluents for chromatography were purchased from Scharlau (Barcelona, Spain), if not stated otherwise. HPLC-grade water was obtained with an EASY-pure II water purification system (Barnstead; Dubuque, IA, USA). Formic acid was from Sigma-Aldrich (Buchs, Switzerland).

Initial screening of the extract library was done as previously described [6]. HPLC based activity profiling: Separations for microfractionation and for on line data collection were carried out on an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column oven and PDA detector (all from Agilent; Waldbronn, Germany). MS spectra were recorded in the range of *m/z* 200–1500 positive and negative mode on an Esquire 3000 Plus ion trap mass spectrometer equipped with an electrospray interface (Bruker Daltonics; Bremen, Germany). Microfractionation was performed with 350 µg of extract in DMSO (10 mg/ml) which were separated on a RP-HPLC SunFire C18 column (3.5 µm, 3×150 mm) (Waters; Wexford, Ireland) with gradient A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid), 10–100% B in 30 min and hold at 100% B for 2 min, at a flow rate of 0.5 ml/min. Hystar 3.2 software (Bruker Daltonics; Bremen, Germany) was used to monitor the HPLC system. An FC 204 fraction collector (Gilson; Middleton, WI, USA) was attached to the HPLC to collect a total of 32 microfractions of 60 s each into 96-deep well plates (Whatman; Florham Park, NJ, USA). The plates were dried in a Genevac EZ-2 Plus™ vacuum centrifuge (Avantec; Ipswich, UK), and microfractions redissolved in 5 µl of DMSO prior to testing for antitrypanosomal activity as described [6].

Semipreparative HPLC for compound isolation was done on an Agilent 1100 series HPLC system consisting of a 1100 series quaternary low-pressure mixing pump with degasser module, column oven, and a 1100 series PDA detector with a 1000 µl loop. A SunFire C18 column (5 µm, 10×150 mm) (Waters) was used. The mobile phase was: A (water) and B (MeOH) with a gradient of 60–100% B in 20 min, followed by flushing 5 min with 100% B, the flow rate was 5 ml/min, and monitored at 220 nm. In each run 15 mg of fraction in 50 µl DMSO was injected.

High resolution HPLC–MS was recorded with an Agilent 1100 series HPLC linked to a micrOTOF-ESI-MS system (Bruker). MS calibration was performed using a reference solution of sodium formate 0.1% in isopropanol–water (1:1) containing 5 mM sodium hydroxide. The typical mass accuracy was ±3 ppm. HyStar 3.0 software (Bruker Daltonics) was used for data acquisition and processing.

NMR experiments were recorded in d_6-DMSO and $CDCl_3$ on a Bruker Avance III spectrometer (Bruker; Fallanden, Switzerland) operating at 500.13 MHz at a target temperature of 18 °C. ^{1}H NMR, COSY, HSQC, and HMBC spectra were measured with a 1 mm TXI probe. The 2D pulse programs were hsqcedetpg, hmbcgplpndqf, and cosygpqf. Spectra processing and analysing was done with Bruker TopSpin 2.1 software.

2.2. Plant material

The dried roots of *S. costus* (MTS 577) were purchased from Peter Weinfurth in 2008 (Bochum, Germany). The leaves of *Laurus nobilis* L. (MTS 693) were purchased from Dixa AG (St. Gallen, Switzerland). The aerial parts of *Eupatorium cannabinum* L. (MTS 771) were harvested near Liestal, Canton Basel Land, Switzerland. The authentication of collected *E. cannabinum* was carried out by Dr. M. Adams. Voucher specimens of all samples are deposited at the Institute of Pharmaceutical Biology, University of Basel, organised under the MTS numbers shown above.

2.3. Extraction and preparative isolation

Dried roots of *S. costus* were finely ground using a ZM1 ultra centrifugal mill (Retsch; Haan, Germany). Maceration of 900 g of the powdered material four times with 4 l of ethyl acetate overnight at room temperature in a glass column 15×26 cm (Pyrex, Ostermundigen, Switzerland) yielded 40 g of a thick brown extract. An aliquot of 30 g of extract was fractionated by open column chromatography (9×59 cm) filled with silica gel (Kieselgel 60™, particle size 40–63 µm) (Merck; Darmstadt, Germany) and isocratic elution with *n*-hexane-ethyl acetate (9:1) at a flow rate of 20 ml/min. Fractions of 500 ml each were collected and monitored with TLC on pre-coated Kieselgel 60 F_{254}, 0.25 mm plates from Merck with a mobile phase of: toluene:ethyl acetate (9:1); detection with vaniline-H_2SO_4. Similar fractions were pooled resulting in 7 fractions. Fraction 5 was obtained as a brown oil (160 mg) and was separated by semi-preparative HPLC as described above to obtain compounds arbusculin B (**1**) (2.5 mg) and α-cyclocostunolide (**2**) (26 mg). Fraction 6 totalling 2.2 g afforded pure costunolide (**3**) (670 mg) and fraction 7 (9.7 g) gave dehydrocostuslactone (**4**) (6.5 g).

Zaluzanin D (**6**) from the ethyl acetate extract of the dried leaves of *Laurus nobilis* L. (Myrtaceae) and eupatoriopicrin (**7**) from the aerial parts of *Eupatorium cannabinum* L. (Asteraceae) were isolated by semipreparative HPLC as described above. Isolated substances **1**–**4**, **6**, and **7** were more than 95% pure according to their ^{1}H NMR spectra. Parthenolide (**5**) was purchased from Alexis Biochemicals (Lausen, Switzerland) with more than 95% pure as stated by the supplier. The compounds **7** were identified by comparison of 1D and 2D ^{1}H NMR, HPLC-HRTOF–MS data with published studies: **1** [12], **2** [13], **3** and **4** [14,15], **6** [16], and **7** [17]. Proton and carbon NMR data of all the isolated compounds are given in the supporting information (Table S1).

2.4. Antitrypanosomal assay

Preparation of *T. b. rhodesiense* STIB 900 stocks and culture media for the bloodstream forms was done according to Baltz et al. [18] with the following modifications: 2-mercaptoethanol 0.2 mM, sodium pyruvate 1 mM, hypoxanthine 0.5 mM, and 15% heat-inactivated horse serum. In vitro screening for antitrypanosomal activity was done with of Alamar Blue® assay [19]. Stock solutions of test compounds and their serial dilutions (5 μl) were transferred into 96 well plates (Costar; Corning Inc., Lowell, MA, USA) containing 50 μl of culture medium per well. Bloodstream forms of STIB 900 in 45 μl of medium were added to each well and the plate was incubated at 37 °C under 5% CO_2 atmosphere for 72 h. Ten microliters of resazurin solution (12.5 mg dissolved in 100 ml distilled water) (Sigma-Aldrich; Zürich, Switzerland) was then added and incubated for further 2–4 h. The fluorescence development was measured in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corp.; Sunnyvale, CA, USA), operating with an excitation wavelength of 536 nm and an emission wavelength of 588 nm and expressed as percentage of the control. The IC_{50} values were calculated in the graphic programme Softmax Pro (Molecular Devices Corp.). Tests were done at least in three independent experiments in duplicate.

2.5. Cytotoxicity assay

Cytotoxicity was determined using rat skeletal myoblast (L6 cells). The culture medium was RPMI 1640 medium supplemented with L-glutamine 2 mM, HEPES 5.95 g/l, $NaHCO_3$ 2 g/l and 10% foetal bovine serum. Podophyllotoxin (Sigma-Aldrich) was used as the reference drug. The assay was performed following the antitrypanosomal assay protocol [19]. The IC_{50}s were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices Corp.). Tests were done in three independent experiments in duplicate.

3. Results and discussion

The preliminary screen of our in house extract library showed the ethyl acetate extract of *S. costus* to be highly active against *T. b. rhodesiense* with 96% inhibition at 4.8 μg/ml. This extract was subjected to HPLC based activity profiling to track the activity and identify the active principles. The activity was almost entirely focused in minutes 22 and 23 with 99% to 100% of inhibition. Fig. 1 shows the overlay of the chromatogram (ESI positive MS trace at scan *m/z* 200–1500) with the percent inhibition of these microfractions. Subsequently, compounds **3** and **4** from these two active windows were isolated.

The major compound **3** (t_R 22.4 min) had a molecular formula of $C_{15}H_{20}O_2$ with *m/z* 465.3182 $[2M+H]^+$ (calcd. for $C_{30}H_{41}O_4$ 465.3006) and was identified as costunolide. Compound **4** (t_R 23.0 min) with a molecular formula of $C_{15}H_{18}O_2$ derived from *m/z* 461.2869 $[2M+H]^+$ (calcd. for $C_{30}H_{37}O_4$ 461.2693) was dehydrocostuslactone. In addition, compounds **1** (t_R 24.2 min) and **2** (t_R 25.3 min) were isolated and both had the sum formula $C_{15}H_{20}O_2$ as suggested by the *m/z* 465.3185 $[2M+H]^+$ (calcd. for $C_{30}H_{41}O_4$ 465.3006) and *m/z* 465.3190 $[2M+H]^+$ (calcd. for $C_{30}H_{41}O_4$ 465.3006). They were identified as the known compounds arbusculin B (**1**) and α-cyclocostunolide (**2**) (Fig. 2).

Compounds **2**, **3**, and **4** have been isolated from *S. costus* previously [20]. Whilst compound **1** is reported here for the first time from this plant.

Further possibly active compounds present in the extract in minor concentrations, like the peaks seen in minute 11 and 20, were not isolated. This may be done in further studies.

From *L. nobilis* we isolated the sesquiterpene lactone **6** and from *Eupatorium cannabium* substance **7**. Compound **6** with sum formula of $C_{17}H_{20}O_4$, *m/z* 311.1289 $[M+Na]^+$ (calcd. for $C_{17}H_{20}O_4Na$ 311.1254) was determined as zaluzanin D and compound **7** with molecular formula of $C_{20}H_{26}O_6$, *m/z* 385.1614 $[M+Na]^+$ (calcd. for $C_{20}H_{26}O_6Na$ 385.1622) was characterised as eupatoriopicrin. Substance **5** was from a commercial source. The ^1H and ^{13}C NMR data

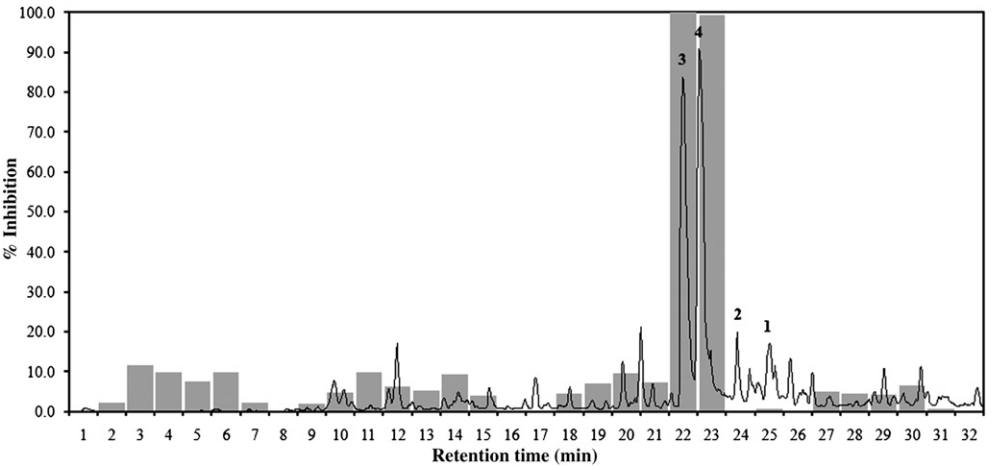

Fig. 1. The antitrypanosomal activity of the 32 one-minute microfractions plotted against the mass trace of the ethyl acetate extract of *Saussurea costus* (ESI-MS positive scan *m/z* 200–1500).

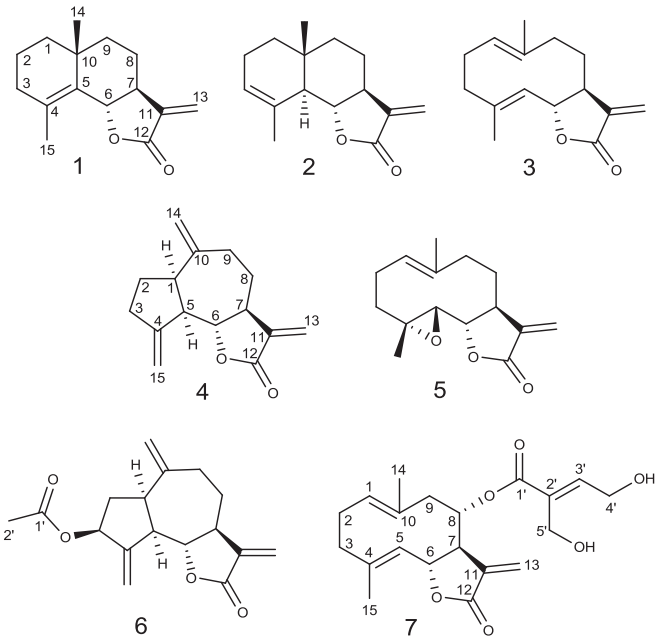

Fig. 2. Chemical structures of seven sesquiterpene lactones isolated from *Saussurea costus*(**1–4**), *Laurus nobilis* (**6**) and *Eupatorium cannabinum* (**7**). Compound **5** was from a commercial source.

for compounds **1–4**, **6**, and **7** are attached as supporting information (Table S1).

Sesquiterpene lactones are classified into several classes based on their carbocyclic skeleton [21]. The compounds in this study are eudesmanolides (6/6 bicyclic), germacranolides (10 member-ring monocyclic), and guaianolides (5/7 bicyclic).

The antitrypanosomal and cytotoxic IC_{50} of the 7 compounds were established (Table 1). The three germacranolides parthenolide (**5**), eupatoriopicrin (**7**), and costunolide (**3**) were the most antitrypanosomally active compounds in this study, followed by the guaianolides dehydrocostuslactone (**4**) and zaluzanin D (**6**), and the eudesmanolides arbusculin B (**1**), and α-cyclocostunolide (**2**). The cytotoxicity of all compounds was within range of IC_{50}s from 1.6 to 19.4 μM and selectivity indices between 0.5 and 6.5.

The three active germacranolides **3**, **5**, and **7** had similar IC_{50} values, suggesting that the presence of the epoxide in **3** or the esterified hydroxyl side chain in **7** did not greatly affect the antitrypanosomal activity. Compounds **3** and **5** were more selective towards *T. b. rhodesiense* than towards mammalian cells (SI: 5.9 and 6.5) whereas **7** was more toxic (SI: 1.3). Amongst the guaianolides, dehydrocostuslactone (**4**) had the threefold activity and twofold toxicity of zaluzanin D (**6**). They both had similar selectivity indices (1.9 and 1.4). The lowest antitrypanosomal activities and selectivities were seen for the two eudesmanolides arbusculin B (**1**) and α-cyclocostunolide (**2**) which were both more active against mammalian cells than against the parasites with selectivity indices of 0.5 and 0.9.

Lirussi et al. [22] report the potent inhibition (IC_{50} = 2.4 μg/ml) of *T. cruzi e*pimastigotes by a methanol extract of *S. costus* roots. Schmidt et al. [23] report parthenolide (**5**) to be highly active against *T. b. rhodesiense* and *T. cruzi*. The study also shows that the tested eudesmanolides tended to have lower antitrypanosomal activities and selectivities than some other types of sesquiterpene lactones. Izumi et al. [24] also previously publish the potent effect of parthenolide (**5**) against *T. cruzi*. The other compounds in this study have so far not been tested for antitrypanosomal effects. We recently reported the potent in vitro and in vivo activity of cynaropicrin, a guaianolide sesquiterpene lactone with a 2-hydroxymethyl-2-propenoyl moiety at C-8, against *T. b. rhodesiense* [9].

Sesquiterpene lactones may exert their various biological activities by the interaction of their α-methylene-γ-butyrolactone moiety with the thiol groups of biomacromolecules through Michael-addition [25].

Table 1
In vitro antitrypanosomal activity and cytotoxicity of sesquiterpene lactones against *T. b. rhodesiense* and cytotoxic effects against L6 cells. Results are expressed as IC_{50} values with the standard deviation (SD) and shown alongside the positive controls.

Compound	*T. b. rhodesiense*	L6 cells	SI
	IC_{50} (μM ± SD)	IC_{50} (μM ± SD)	
1 Arbusculin B	12.0 ± 3.3	6.2 ± 2.3	0.5
2 α-Cyclocostunolide	21.9 ± 1.1	19.4 ± 7.9	0.9
3 Costunolide	1.3 ± 0.4	7.7 ± 1.3	5.9
4 Dehydrocostuslactone	4.4 ± 1.4	8.3 ± 1.9	1.9
5 Parthenolide	0.8 ± 0.5	5.2 ± 0.9	6.5
6 Zaluzanin D	10.8 [a]	15.6 [a]	1.4
7 Eupatoriopicrin	1.2 ± 0.2	1.6 ± 0.08	1.3
Melarsoprol	0.006 ± 0.003		
Podophyllotoxin		0.01 ± 0.002	

[a] IC_{50} value is obtained from a single experiment done in duplicate.

A comprehensive study on 40 sesquiterpene lactones from five sesquiterpene lactone classes confirms the influence of this α-methylene-γ-butyrolactone moiety to their antitrypanosomal and cytotoxic activity. The most potent and selective sesquiterpene lactones reported so far were the pseudoguaianolide helenalin and the xanthanolide 8-epixanthatin-1,5-epoxide. However, a high degree of correlation is also found between such compounds' antiprotozoal activity and mammalian cytotoxicity so that it may be difficult to find a structural explanation for the selectivity observed with some compounds [23]. Cytotoxicity studies of sesquiterpene lactones related to the lactone moiety were shown by Kupchan et al. [26,27].

This study suggests that sesquiterpene lactones may have potential for the development of new leads to treat infection caused by trypanosomes. Further comprehensive structure activity studies to clarify the contribution of sesquiterpene ring systems and/or substitution patterns of sesquiterpene lactones to the overall activity against both *T. b. rhodesiense* and mammalian cell like those reported by Schmidt et al. [23] and especially further in vivo data like Zimmermann et al. [9] are needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2011.05.010.

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Supporting Information

Antitrypanosomal sesquiterpene lactones from *Saussurea costus*

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S Table 1. ^{13}C (125 Mhz) and ^{1}H (500 MHz) data for compounds **1**-**4** in DMSO-d_6 and compounds **6** and **7** in $CDCl_3$ (δ in ppm and *J* in Hz). Assignment of ^{13}C shifts was done via HSQC and HMBC correlations.

Position	1		2		3		4		6		7	
	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
1	41.3, CH_2*	1.58, m; 1.41, m	21.5, CH_2	1.62, m; 1.99, m	126.7, CH	4.82, dd (11.3, 4.3)	47.0, CH	2.95, m	50.3, CH	2.93, m	130.7, CH	4.87, dd (2.8, 11.0)
2	18.8, CH_2*	1.57, m	23.0, CH_2	2.04, m	26.1, CH_2	2.27, m; 2.10, m	30.2, CH_2	1.89, m; 1.82, m	36.0, CH_2	2.43, m; 1.82, m	26.1, CH	2.31, m; 2.20, m
3	31.0, CH_2*	2.00, m; 1.89, m	122.5, CH	5.37, brs	39.3, CH_2	2.25, m; 1.98, m	32.6, CH_2	2.48, m	74.9, CH	5.57, m	39.4, CH_2	2.35, m; 2.07, m
4	139.7, qC		133.2, qC		141.0, qC		152.3, qC		148.9, qC		142.5, qC	
5	130.0, qC		50.7, CH	2.35, brd	127.8, CH	4.77, t (9.7)	52.0, CH	2.86, dd (10.0, 8.8)	44.2, CH	3.01, m	127.2, CH	4.76, d (10.1)
6	83.2, CH	4.61, brd (11.5)	82.0, CH	3.94, t (11.2)	81.7, CH	4.71, t (9.8)	85.0, CH	3.98 t (9.2)	84.5, CH	4.10, dd (9.4, 9.4)	75.4, CH	5.16, dd (9.0, 9.7)
7	50.0, CH	2.55, m	51.0, CH	2.59, m	50.0, CH	2.63, m	44.6, CH	2.97, m	45.1, CH	2.97, m	52.9, CH	2.93, m
8	23.0, CH_2	2.06, m; 1.63, m	39.0, CH_2	1.52, m; 1.39, m	27.7, CH_2	2.12, m; 1.69, m	30.8, CH_2	2.25, m; 1.33, m	30.4, CH_2	2.01, m; 1.44 m	72.3, CH	5.80, d (3.2)
9	40.6, CH_2*	1.50, m; 1.36, m	37.5, CH_2	1.45, m; 1.36, m	40.9, CH_2	2.33, m; 2.08, m	36.2, CH_2	2.43, m; 2.14, m	34.4, CH_2	2.51, m; 2.21, m	44.2, CH_2	2.84, m; 2.32, m
10	37.7, qC		23.3, qC		137.3, qC		150.2, qC		149.0, qC		134.0, qC	
11	127.0, qC		139.8, qC		141.2, qC		140.2, qC		139.8, qC		136.8, qC	
12	170.3, qC		170.7, qC		170.4, qC		169.8, qC		171.0, qC		170.6, qC	
13	118.4, CH_2	6.00, d (3.1); 5.56, d (3.0)	116.5, CH	5.91, d (3.1); 5.48, d (3.1)	119.6, CH_2	6.07, d (3.6) 5.67, d (3.3)	120.1, CH_2	6.09, d (3.6); 5.66, d (3.2)	119.0, CH_2	6.13, d (3.5); 5.59, d (3.2)	120.9, CH_2	6.29, d (3.5); 5.57, d (3.0)
14	27.2, CH_3	1.09, s	17.5, CH_3	0.86, s	16.1, CH_3	1.39, s	112.3, CH_2	4.89, brs; 4.79, brs	112.8, CH_2	4.96, s	17.5, CH_3	1.46, s
15	20.6, CH_3	1.80, s	23.8, CH_3	1.76, s	17.3, CH_3	1.66 s	108.6, CH_2	5.13, brs; 5.03, brs	111.5, CH_2	5.38, m; 5.23,m	19.0, CH_3	1.75, s
1′									170.5, qC		165.9, qC	
2′									19.6, CH_3	2.10, s	131.8, qC	
3′											144.7, CH	6.84, dd (5.7, 5.7)
4′											58.9, CH_2	4.40, d (5.9)
5′											57.3, CH_2	4.33, s

* These signals may be interchangeable.

CHAPTER IV

HPLC-BASED ACTIVITY PROFILING FOR ANTIPLASMODIAL COMPOUNDS IN THE TRADITIONAL INDONESIAN MEDICINAL PLANT *CARICA PAPAYA* L.

HPLC-based activity profiling for antiplasmodial compounds in the traditional Indonesian medicinal plant *Carica papaya* L.

Tasqiah Julianti, Maria de Mieri, Stefanie Zimmermann, Samad Ebrahimi, Marcel Kaiser, Markus Neuburger, Melanie Raith, Reto Brun, Matthias Hamburger. *Journal of Ethnopharmacology*, submitted.

Methanolic extract of papaya leaves inhibited the growth of *Plasmodium falciparum in vitro*. Active compounds within the extract were localized by HPLC-based activity profiling. Following HPLC and MPLC semipreparatively isolation and then structures elucidation by microprobe NMR spectroscopy and high resolution mass spectrometry, nine compounds were obtained. Absolute configuration of two compounds was performed by X-ray crystallography along with calculated and experimental ECD. When tested *in vitro*, four flavonols were moderately active, two monomeric piperidine alkaloids were inactive, and three dimeric piperidine alkaloids were highly active. In this study, carpaine was the most active compound with remarkable selectivity to rat myoblast L-6 cells. Further *in vivo* tested of carpaine using mouse model applying the malaria 4-day suppressive test, however, displayed inactivity.

Extraction of plant material, development of alkaloidal fractionation method, HPLC-microfractionation, recording and data interpretation for HPLC-based activity profiling, recording and data interpretation for structure elucidation by HPLC-ESI-TOF-MS; microprobe NMR; optical rotation; experimental ECD, draft writing and partly figure preparation for the manuscript are my contributions to this publication.

Tasqiah Julianti

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Research Paper

HPLC-based activity profiling for antiplasmodial activity in the traditional Indonesian medicinal plant *Carica papaya* L.

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ABSTRACT

Ethnopharmacological relevance: Leaf decoctions of *Carica papaya* have been traditionally used in some parts of Indonesia to treat and prevent malaria. Leaf extracts and fraction have been previously shown to possess antiplasmodial activity *in vitro* and *in vivo*.
Materials and methods: Antiplasmodial activity of extracts was confirmed and the active fractions in the extract were identified by HPLC-based activity profiling, a gradient HPLC fractionation of a single injection of the extract, followed by offline bioassay of the obtained microfractions. For preparative isolation of compounds, an alkaloidal fraction was obtained *via* adsorption on cationic ion exchange resin. Active compounds were purified by HPLC–MS and MPLC–ELSD. Structures were established by HR-ESI-MS and NMR spectroscopy. For compounds **5** and **7** absolute configuration was confirmed by comparison of experimental and calculated electronic circular dichroism (ECD) spectroscopy data, and by X-ray crystallography. Compounds were tested for bioactivity *in vitro* against four parasites (*Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*), and in the *Plasmodium berghei* mouse model.
Results: Profiling indicated flavonoids and alkaloids in the active time windows. A total of nine compounds were isolated. Four were known flavonols – manghaslin, clitorin, rutin, and nicotiflorin. Five compounds isolated from the alkaloidal fraction were piperidine alkaloids. Compounds **5** and **6** were inactive carpamic acid and methyl carpamate, while three alkaloids **7**–**9** showed high antiplasmodial activity and low cytotoxicity. When tested in the *Plasmodium berghei* mouse model, carpaine (**7**) did not increase the survival time of animals.
Conclusions: The antiplasmodial activity of papaya leaves could be linked to alkaloids. Among these, carpaine was highly active and selective *in vitro*. The high *in vitro* activity could not be substantiated with the *in vivo* murine model. Further investigations are needed to clarify the divergence between our negative *in vivo* results for carpaine, and previous reports of *in vivo* activity with papaya leaf extracts.

1. Introduction

Based on the number of cases reported, malaria is considered as a controlled disease in Indonesia (Feachem et al., 2010). However, malaria is still a major health concern in the densely forested parts of eastern Indonesia. Current primary treatment for malaria is Artemisinin Combination Therapies (ATCs), but artemisinin-resistant *Plasmodium falciparum* strains have been reported (Miller et al., 2013). Thus, a continued effort for discovery of novel antimalarial compounds is needed.

Traditional remedies to prevent and treat malaria remain in use in Indonesia. Among these, papaya leaf decoctions are widely used in Papua and Maluku islands. Other than malaria therapy, papaya leaves are empirically used in Indonesia to enhance breast milk production, for deworming and boosting appetite, and for reducing fever (Rehena, 2009; Sastroamidjojo, 2001; Tjahjadi, 1990). Use of papaya leaves as an antimalarial remedy has also been reported from India, and from some Latin American and African

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countries (Asasea et al., 2010; Kovendan et al., 2012; Stangelanda et al., 2011; Valadeau et al., 2009). The traditional use as an antimalarial remedy was substantiated by *in vitro* studies (Lina, 1996; Rehena, 2009), and by testing of an alkaloid containing polar leaf extract in a mouse model (Sulistyowati, 2000).

Papaya (*Carica papaya* L., Caricaceae) grows widely in tropical and subtropical regions around the world (Garrett, 1995). The trees are mainly cultivated for their fruits, but the leaves, seeds, and latex are traditionally known to benefit health. Papaya leaves contain flavonoids and other phenolic compounds, saponins, cardiac glycosides, anthraquinones, and alkaloids (Afzan et al., 2012; Canini et al., 2007; Sherwani et al., 2013). Alkaloids reported include carpaine (Greshoff, 1890), pseudocarpaine (Govindachari et al., 1954), and dehydrocarpaine I and II (Tang, 1979).

Considering the easy accessibility of plant material, the traditional use as an antiplasmodial, and the reported *in vivo* activity, we embarked on an activity-driven characterization of active principles in papaya leaves. For an efficient localization of active constituents in the extract we employed HPLC-based activity profiling (Potterat and Hamburger, 2013), using a protocol established for the discovery of antiprotozoal compounds in complex matrices (Adams et al., 2009). Analytical HPLC connected to PDA and MS detectors, and parallel micro-fractionation of the column effluent for off-line bioassay link biological and structural information with chromatographic peaks in the chromatogram.

We here report on the identification of *in vitro* antiplasmodial compounds in papaya leaves, and on the outcomes from a testing of the main alkaloid, carpaine, in the murine *Plasmodium berghei* model.

2. Materials and methods

2.1. Materials

Analytical grade solvents for extraction and HPLC grade solvents were from Scharlau (Barcelona, Spain). Ammonium hydroxide (26%) was from Riedel-de Häen (Seelze, Germany). Formic acid (98–100%) was from Sigma-Aldrich (Buchs, Switzerland). DMSO was from Reuss Chemie (Tägerig, Switzerland). HPLC grade water was obtained by an EASYpure II (Barnstead, Dubuque, USA) water purification system. Cationic exchange resin Lewatit® MonoPlus SP 112 was from Lanxess (Cologne, Germany). Reference drugs for bioactivity tests were melarsoprol (Arsobal®, Sanofi-Aventis, Switzerland), benznidazole (Sigma-Aldrich), miltefosine (VWR), chloroquine (Sigma-Aldrich), artesunate (Mepha, Switzerland), and podophyllotoxin (Sigma-Aldrich).

Parasites for *in vitro* activity tests were *Trypanosoma brucei rhodesiense*, STIB 900 strain, trypomastigotestage; *Trypanosoma cruzi*, Tulahuen C4 strain, amastigote stage; *Leishmania donovani*, MHOM-ET-67/L82 strain, amastigote stage; *Plasmodium falciparum*, K1 strain (chloroquine- and pyrimethamine-resistant), erythrocytic stage. Cytotoxicity was determined with rat skeletal myoblast cells (L6). The *in vivo* efficacy study was carried out in the *Plasmodium berghei* mouse model. Adult female NMRI mice were purchased from RCC Janvier.

2.2. General experimental procedures

Extract screening and HPLC-based activity profiling including mass spectral data analysis were carried out as previously described by Adams et al. (2009). A HPLC SunFire RP-18 column (3.5 μm, 3 × 150 mm^2 i.d., Waters; Wexvord, Ireland) was used. Minute-based microfractination and offline data collection for HPLC based activity profiling were carried out with a series 1100 HPLC system consisting of a degasser, a binary high pressure mixing pump, a column oven and a PDA detector with 250 μL loop (all from Agilent; Waldbronn, Germany) connected to an Esquire 3000 Plus ion trap mass spectrometer with an electrospray interface (Bruker Daltonics; Bremen, Germany). MS spectra were recorded in positive and negative mode in the range of *m*/*z* 200–1500. Hystar 3.0 software (Bruker Daltonics; Bremen, Germany) was used for controlling the LC–MS system.

Semipreparative HPLC separations of flavonoids and alkaloid **9** were carried out on an 1100 series HPLC system consisting of a quaternary low-pressure mixing pump with a degasser module, a column oven, and a PDA detector with a 1000 μL loop (all Agilent; Waldbronn, Germany). A SunFire C18 column (5 μm, 10 × 150 mm^2; Waters) was used. The separation of flavonoids was monitored following UV-detection. For the alkaloid, the separation was monitored using an Esquire 3000 Plus ion trap mass spectrometer with an electrospray interface (Bruker Daltonics; Bremen, Germany) connected *via* a T-splitter (split ratio 3:997). Data analysis and controlling of the MS were with Hystar 3.2 software (Bruker Daltonics; Bremen, Germany). Spectra were recorded in positive mode in the range of *m*/*z* 200–1000.

Semipreparative separations of alkaloids **5**–**8** were carried out with a PuriFlash 4100 system consisting of a mixing HPLC pump, a UV detector dual length DAD, a fraction collector, and a sample loading module (Interchim; Montluçon, French). For system controlling and process monitoring, Interchim Software 5.0 was used. Detection was done with a 2000ES ELSD (Alltech; Woodridge, Illinois, USA). The following ELSD settings were used: temperature 60 °C, gas flow 2.4 L/min, and gain of 4 or 8, with impactor on.

High resolution MS were recorded with an Agilent 1100 series HPLC linked to a microTOF-ESI-MS system (Bruker Daltonics). HyStar 3.0 software (Bruker Daltonics) was used for data acquisition and processing.

NMR spectra were recorded in CD_3OD and $CDCl_3$ on a Bruker Avance III spectrometer (Bruker; Fällanden, Switzerland) operating at 500.13 MHz for ^1H, and 125.77 MHz for ^{13}C. ^1H NMR and 2D (COSY, HSQC, and HMBC) spectra were measured with a 1 mm TXI probe at 18 °C. ^{13}C NMR spectra were recorded with a 5 mm BBO probe at 23 °C. Spectra were processed and analyzed by Bruker TopSpin 3.0 software.

ECD spectra of compounds were recorded, at 500 μg/mL in MeOH or MeCN, on an AVIV Model 62ADS CD spectrometer, and analyzed with the AVIV 60DS V4.1 software.

X-ray crystallography was performed with a Bruker Kappa Apex2 diffractometer at 123 K using graphite-monochromated Cu K_α-radiation. Structure solution used program SIR92 (Altomare et al., 1994) and SHELX 86 (Sheldrick, 1985). Structure refinement employed CRYSTALS (Betteridge et al., 2003). Data analysis and visualization utilized Mercury v.3.0 software.

2.3. Plant material and extraction

Extract screening and HPLC-based activity profiling were carried out with a leaf sample purchased from a Thai food store in Basel. Isolation of compounds was carried out with papaya leaves purchased from Dixa AG (St. Gallen, Switzerland), a supplier of pharmaceutical herbs. Vouchers have been deposited under identification nos. 910 and 647 at the Division of Pharmaceutical Biology, University of Basel, Switzerland. Authentication of the material was carried out by M. Hamburger.

In the initial screening, the extracts of methanol, ethyl acetate, and petroleum ether were prepared by pressurized liquid extraction using an ASE 200 extractor with solvent module (Dionex; Sunnyvale, CA, USA). Extraction was performed with 1 g of ground leaves in a 22-mL cartridge. Instrument setting for three cycles extraction were of temperature 100 °C, preheating time 1 min, heating time 5 min, static extraction 5 min, flush 100% solvent of cell volume, purge 120 s with nitrogen, and pressure 120 bar. The

same methanolic extract was used for minute-based microfractionation with HPLC for activity and chemical profiling.

For isolation of flavonoids, an aliquot of coarsely minced leaves (300 g) were macerated with MeOH (4 L). Evaporation of solvent afforded 31 g of dark green extract A.

For preparative isolation of alkaloids, coarsely powdered papaya leaves (1 kg) were moistened with 680 mL of MeOH–NH_4OH conc. (1:1). The leaf material was then packed into a percolator (5×80 cm^2 i.d.) and extracted with n-hexane (approx. 12 L). Evaporation under reduced pressure yielded 56 g of a dark green extract B.

2.4. Isolation of compounds *1–4*

Extract A (20 g) was separated on a Sephadex LH-20 column (9.5×27 cm^2 i.d.) with MeOH as eluent. The eluate was analyzed by HPLC–PDA–MS (at 254 nm and ESI positive ion mode) using the standard LC method described by Adams et al. (2009), and 10 fractions were obtained. Fractions 5 (1.2 g) and 8 (0.4 g) were separated by semipreparative HPLC with UV-detection at 254 nm. The mobile phase was 0.1% aqueous formic acid (A) and 0.1% formic acid in MeCN (B), running on gradient of 10–40 B in 35 min, stay at 40% B for 3 min, 40–10% in 2 min for washing, and flow rate of 5 mL/min. For each run, an aliquot of 100 μL of solution (15% in MeOH) was injected. We obtained compounds **1** (2.1 mg), **2** (4.4 mg), **3** (22.2 mg), and **4** (12.6 mg), respectively.

2.5. Isolation of compounds *5–8*

An aliquot (45 g) of extract B was sonicated with 1000 mL methanol and centrifugated for 10 min at 3000 rpm, (Megafuge 2.0 R, Heraeus; Schwerte, Germany) to obtain a clear solution. The solution was acidified with 50 mL HCl 1 N and filtered. The filtrate was applied onto a column (5×80 cm^2 i.d.) packed with 500 g Lewatit® MonoPlus SP 112 resin (Lanxess; Leverkusen, Germany). Prior to that, the resin was conditioned with 4×500 mL MeOH, and 4×500 mL of MeOH–HCl 1 N (20:1). The column was washed with MeOH–HCl 1 N (20:1) to remove the non-alkaloidal constituents, and with MeOH–NH_4OH conc. (20:1) to collect the alkaloidal fraction. The effluents were collected and monitored by LC–MS. The amount of alkaloidal fraction was 1.9 g.

The alkaloidal fraction was separated on a C18 cartridge (86 g, RediSep®Rf; Teledyne Isco, Nebraska, USA), using a PuriFlash 4100 system (Interchim) connected to a ELSD. The alkaloidal fraction was dissolved as 5% solution in MeCN–H_2O (3:7), and aliquots of 2 mL were injected. The mobile phase for separation was H_2O (A) and acetonitrile (B), both containing 0.1% formic acid. The following gradient was used: 10–30% B in 30 min, followed by 30–100% for 30–60 min. The flow rate was 60 mL/min. Four fractions were collected based on retention time and ELSD trace – 1 (137 mg, t_R min 9–14), 2 (160 mg, t_R min 14–18), 3 (336 mg, t_R min 18–25), and 4 (170 mg, t_R min 25–29).

Each of these fractions was further separated with the LC–ELSD system above. Fractions 1–3 were chromatographed on a C18 cartridge (43 g; RediSep®Rf) under isocratic conditions (10% B for purification of **5**), 12% B for **6**, and 15% B for **7**. The flow rate was 30 mL/min. Fraction 4 was separated on a HP C18 cartridge (30 g; RediSep Rf Gold®) with a gradient of 10–30% B over 30 min, at a flow rate of 30 mL/min, to afford **8**. Overall, 65 mg of compound **5**, 93 mg of **6**, 44 mg of **7**, and 21 mg of **8** were obtained. Purity of compounds was ≥96% as determined by LC–ESIMS in positive ion mode.

2.6. Isolation of compound *9*

An aliquot of 6 g of n-hexane extract was filtered through a MonoPlus SP 112 resin column using EtOAc–MeOH–HCl 0.1 M (7:2:1) and EtOAc–MeOH–conc. NH_4OH (7:2:1) as eluents. After evaporation of the basic eluent under reduced pressure, 16 g of voluminous white granules were obtained. The solid residue was suspended in 1.8 L 1 M NH_4OH solution (pH 11) and partitioned with 3×2 L ether. Removal of ether under reduced pressure yielded 179 mg of a residue, which was dissolved in 5 mL of aqueous MeCN (30%, v/v, 0.1% formic acid) and separated by semi-preparative HPLC using MS detection. Aliquots of 400 μL solution (3% in MeOH) were injected for each run. The mobile phase was 0.1% aqueous formic acid (A) and 0.1% formic acid in MeCN (B) running in gradient of 14–18% B in min 0–4, 18% B min 4–6, 18-20% B min 6–7, 20–100% B in 3 min for wash, and flow rate of 5 mL/min. Compound **9** (5 mg) was obtained, along with additional **7** (110 mg) and **8** (9 mg).

2.7. Characterization of compounds

Structures of **1–9** were established with the aid of ESIMS, 1D and 2D NMR data and by comparison with literature data. For selected compounds, X-ray crystallographic analysis and ECD measurements were performed.

8-[(*2′R-5′S-6′S*)-5′-Hydroxy-6′-methylpiperidin-2′-yl]-octanoic acid ((+)-carpamic acid (**5**)): white solid; $[\alpha]_D^{24}$ +6.12 (c 0.13 MeOH) Ref. +6.0 (c 0.4 MeOH) (Masuda et al., 2006); HR-ESI-MS: *m/z* 258.2047 $[M+H]^+$ (calcd. for $C_{14}H_{28}NO_3$: 258.2064); ^1H NMR (500 MHz, CD_3OD) δ 1.25–1.44 (8H, m, CH_2-4, 5, 6, 7), 1.31 (3H, d, J=6.6 Hz, CH_3-6′), 1.44–1.78 (7H, m, CH_2-3, -8, -3′, H_{ax}-4′), 1.96 (1H, dt, J=9.5 and 3.3 Hz, H_{eq}-4′), 2.13 (2H, t, J=7.2 Hz, CH_2-2), 2.96 (1H, m, $W_{1/2}$=14.0 Hz, H-2′), 3.16 (1H, qd, J=6.7 and 1.3 Hz, H-6′), 3.77 (1H, ddd, J=3.5, 1.8 and 1.7 Hz, H-5′); ^{13}C NMR (125 MHz, CD_3OD) δ 16.0 (CH_3, C-6′), 23.7 (CH_2, C-3′), 26.2 (CH_2, C-7), 27.5 (CH_2, C-3), 30.1–30.2–30.5–31.1 (CH_2, C-4, C-5, C-6, C-4′), 34.6 (CH_2, C-8), 38.9 (CH_2, C-2), 57.4 (CH, C-6′), 58.5 (CH, C-2′), 66.0 (CH, C-5′), 183.7(C, C-1).

Methyl-[8-(*2′R-5′S-6′S*)-(5′-hydroxy-6′-methylpiperidin-2′-yl)]-octanoate ((+)-methyl carpamate (**6**)): white solid; $[\alpha]_D^{24}$ +4.01 (c 0.08 MeOH); HR-ESI-MS: *m/z* 272.2207 $[M+H]^+$ (calcd. for $C_{15}H_{30}NO_3$: 272.2220); ^1H NMR (500 MHz, D_2O) δ 1.43–1.63 (8H, m, CH_2-4, -5, -6, -7), 1.47 (3H, d, J=6.6 Hz, CH_3-6′), 1.68–1.88 (5H, m, CH_2-3, -8, H_{ax}-3′), 1.92 (1H, tt, J=14.0 and 3.3 Hz, H_{ax}-4′), 2.02 (1H, dddd, J=14.0, 3.8, 3.5 and 3.0 Hz, H_{eq}-3′), 2.16 (1H, dddd, J=14.4, 3.5, 3.5 and 3.0 Hz, H_{eq}-4′), 2.55 (2H, t, J=7.3 Hz, CH_2-2), 3.28 (1H, m, $W_{1/2}$=14.5 Hz, H-2′), 3.47 (1H, br q, J=6.7 Hz, H-6′) 3.86 (3H, s, CO_2CH_3), 4.14 (1H, br s, H-5′); ^{13}C NMR (125 MHz, CD_3OD) δ 16.0 (CH_3, C-6′), 23.7 (CH_2, C-3′), 26.0–26.3 (CH_2, C-3 and C-7), 30.0–30.1–30.3–31.1 (CH_2, C-4, C-5, C-6, C-4′), 34.7 (CH_2, C-8), 34.8 (CH_2, C-2), 52.1 (CH_3, CO_2CH_3), 57.4 (CH, C-6′), 58.6 (CH, C-2′), 66.0 (CH, C-5′), 175.9 (C, C-1).

(*1S-11R-13S-14S-24R-26S*)-13,26-Dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}]triacontane-3,16-dione ((+)-carpaine (**7**)): white solid/colorless crystal; $[\alpha]_D^{24}$ +25 (c 0.11 EtOH) Ref. +20.9 (Sato et al., 2003); HR-ESI-MS: *m/z* 479.3831 $[M+H]^+$ (calcd. for $C_{28}H_{51}N_2O_4$: 479.3849); ^1H NMR (500 MHz, $CDCl_3$) δ 1.09 (6H, d, J=6.4 Hz, CH_3-11, -11′), 1.16–1.42 (20H, m, CH_2-4, -5, -6, -7, -14, -4′, -5′, -6′, 7′, 14′), 1.40–1.72 (10H, m, CH_2-3, CH_2-8, H_{ax}-13, CH_2-3′, CH_2-8′, H_{ax}-13′), 1.92 (2H, dddd, J=14.0, 3.8, 3.5 and 3.0 Hz, H_{eq}-13, H_{eq}-13′), 2.34 (4H, t, J=7.3 Hz, CH_2-2, -2′), 2.75 (2H, m, $W_{1/2}$=14.5 Hz, H-9,-9′), 3.06 (2H, br q, J=6.7 Hz, H-11, -11′), 4.84 (2H, br s, H-12, -12′).^{13}C NMR (125 MHz, $CDCl_3$) δ 15.8 (CH_3, C-11, C-11′), 23.1(CH_2, C-7, C-7′), 23.3 (CH_2, C-3, C-3′), 25.2 (CH_2, C-14, C-14′), 26.6 (CH_2, C-13, C-13′), 27.7–27.8–28.2 (CH_2, C-4, C-5, C-6, C-4′, C-5′, C-6′), 31.9 (CH_2, C-2, C-2′), 34.1 (CH_2, C-8, C-8′), 53.8 (CH, C-11, C-11′), 56.6 (CH, C-9, C-9′), 68.1 (CH, C-12, C-12′), 172.8 (C, C-1, C-1′).

6-(8-Methoxy-8-oxooctyl)-2-methylpiperidin-3-yl 8-(5-hydroxy-6-methylpiperidin-2-yl)octanoate (**8**): white amorphous solid; $[\alpha]_D^{24}$

+3.09 (c 0.08 $CHCl_3$); HR-ESI-MS: *m/z* 511.4085 $[M+H]^+$ (calcd. for $C_{29}H_{55}N_2O_5$: 511.4111); ^1H NMR (500 MHz, $CDCl_3$) δ 1.06 (3H, d, *J*=6.3 Hz, CH_3-11′), 1.13–1.78 (33H, overlapped), 1.92 (1H, t, *J*=15.0 Hz, H_{ax}-13′), 1.93 (1H, t, *J*=15.0 Hz, H_{ax}-13), 2.23 (2H, t, *J*=7.3 Hz, CH_2-2′), 2.31 (2H, m, CH_2-2), 2.61 (1H, br m, $W_{1/2}$=13.0 Hz, H-9′), 2.78 (1H, br m, $W_{1/2}$=15.0 Hz, H-9), 2.95 (1H, br q, *J*=6.5 Hz, H-11′), 3.01 (1H, br m, H-11), 3.60 (3H, s, $-OCH_3$), 3.70 (1H, br s, H-12), 4.84 (1H, br s, H-12′). ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.6 (CH_3, C-11), 16.5 (CH_3, C-11″), 22.1–24.6–24.7–24.9–25.6–25.8 (CH_2, C-3, C-3′, C-7, C-7′, C-14, C-14′), 27.9–28.3–28.4–28.8–28.9–29.0–29.1–30.7 (CH_2, C-4, C-4′, C-5, C-5′, C-6, C-6′, C-13, C-13′), 33.6–33.9 (CH_2, C-2, C-2′,C-8), 34.2 (CH_2, C-8″), 51.5 (CH_3, $-OCH_3$), 54.3 (CH, C-11′), 56.1 (CH, C-11), 57.0 (CH, C-9′), 57.1 (CH, C-9), 66.2 (CH, C-12), 68.3 (CH, C-12′), 173.1 (C, C-1), 174.2 (C, C-1′).

13,26-Dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}] triacontane-3,16-dione (**9**): white solid; $[\alpha]_D^{24}$ +7.07 (c 0.23 EtOH) Ref. +4.95 (Topuriya et al., 1978); HR-ESI-MS: *m/z* 479.3812 $[M+H]^+$ (calcd. for $C_{28}H_{51}N_2O_4$: 479.3849); ^1H NMR (500 MHz, $CDCl_3$) δ 1.03 (3H, d, *J*=6.3 Hz, CH_3-11), 1.07 (3H, d, *J*=6.5 Hz, CH_3-11′), 1.12–2.00 (32H, overlapped), 2.24–2.41 (4H, m, CH_2-2, CH_2-2′), 2.53 (1H, m, H-9), 2.81–2.92 (2H, m, H-11, H-9′), 3.20 (1H, m, H-11′), 4.76–4.90 (2H, br m, H-12, H-12′). ^{13}C NMR (500 MHz, HSQC/HMBC, $CDCl_3$) δ 15.7 (CH_3, C-11′), 18.4 (CH_3, C-11), 24.5 (CH_2, C-14′), 25.0–25.5 (CH_2, overlapped), 26.6 (CH_2, C-14), 27.1 (CH_2, C-13′), 28.2–29.4 (CH_2, overlapped), 29.3 (CH_2, C-13), 32.6 (CH_2, C-8), 34.8–35.2 (CH_2, C-2, C-2′), 37.2 (CH_2, C-8′), 48.1 (CH, C-11′), 48.3 (CH, C-9′), 53.8 (CH, C-11), 56.5 (CH, C-9), 69.9 (CH, C-12), 71.3 (CH, C-12′), 173.5–173.1 (C, C-1, C-1′)

2.8. Conformational analysis, geometrical optimization, and ECD calculation

Conformational analysis of **7** was performed with Schrodinger MacroModel 9.1 software using the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O. Conformers occurring within a 2 kcal/mol energy window from the global minimum were chosen for geometrical optimization and energy calculation using the density function theory (DFT) with the B3LYP functional and the 6-31G** basis-set in the gas-phase with the Gaussian 09 program (Frisch et al., 2009). Vibrational analysis was done at the same level to confirm minima. TD-DFT/B3LYP/6-31G* in the gas phase and in MeCN using the SCRF method, with the CPCM model. ECD curves were obtained on the basis of rotator strengths with a half-band of 0.2 eV using SpecDis v1.63 (Bruhn et al., 2013). The spectra were combined after Boltzmann weighting according to their population contribution.

2.9. X-ray crystallography

Needles of **5** were obtained from a mixture of MeOH–H_2O kept in the refrigerator for 3 weeks. Needles of **7** were formed upon slow evaporation, at room temperature, of a solution in MeOH–H_2O (50:50).

A suitable crystal of **5** was measured on a Bruker Kappa Apex2 diffractometer. The structure was solved by direct methods using the program SIR92 (Altomare et al., 1994). Least-squares refinement against *F* was carried out on all non-hydrogen atoms using the program CRYSTALS (Betteridge et al., 2003). Chebychev polynomial weights (Prince, 1982; Watkin, 1994) were used to complete the refinement. Plots were produced using CAMERON (Watkin et al., 1996).

The crystal of compound **7** was measured on a Bruker Kappa Apex2 diffractometer. The Apex2 suite (Bruker Manual, 2006) was used for data collection and integration. The structure was solved by direct methods using the program SHELXS 86 (Sheldrick, 1985). Least-squares refinement against *F* was carried out on all non-hydrogen atoms using the program CRYSTALS (Betteridge et al., 2003). Chebychev polynomial weights (Prince, 1982; Watkin, 1994) were used to complete the refinement. Plots were produced using CAMERON (Watkin et al., 1996). Detailed experimental data are provided in Supplementary material (Tables S2 and S3).

2.10. Bioassays

Assays for *in vitro* activity against *Plasmodium falciparum*, *Trypanosoma brucei rhodesiense*, and *in vivo* activity against *Plasmodium berghei* were carried out according to the procedure described by Witschel et al. (2012). The cytotoxicity *in vitro* was conducted following the Alamar Blue assay (Page et al., 1993). All protocols and procedures used in this study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland (authorization no. 739; 11.12.2009). The IC_{50} values are calculated by linear regression (Huber and Koella, 1993).

The *in vivo* test was performed on four NMRI female mice infected with *Plasmodium berghei* ANKA (2×10^7 parasites/mice). The compound was administered intraperitoneally for four consecutive days, at a daily dose of 5 mg/kg. The volume of injection was 10 mL/kg of a clear solution in 10% aqueous DMSO. If no activity was observed (reduction of parasitemia $<40\%$) then the animal was euthanized after determination of parasitemia. Control mice usually would die in day 6–7 after infection. Lethal dose of compound **7** on uninfected mice was determined at 50 mg/kg, administered as injection of 20 mg/kg, and followed by 30 mg/kg after 2 h. The volume of injection was 10 mL/kg.

3. Results and discussion

When tested at 4.81 μg/mL, the EtOAc and MeOH extracts inhibited growth of *Plasmodium falciparum* by 61.03% and 51.85%, respectively (Julianti et al., 2013). Active constituents in the extracts were tracked with the aid of HPLC-based activity profiling (Potterat and Hamburger, 2013). An activity profile of 32 one-minute fractions, and the corresponding LC–PDA–MS traces are shown in Fig. 1. Major inhibition was observed in the time window of minutes 6–12. In the chromatogram recorded with the PDA detector, four well-separated major and three minor peaks were detected within the activity window. In contrast, MS detection (positive polarity) revealed a complex pattern of peaks, whereby molecular ions of 475.3, 477.4, and 479.4 suggested the possible presence of previously reported alkaloids dehydrocarpaine II, dehydrocarpaine I, and carpaine respectively.

We first isolated the four major UV-absorbing compounds **1–4** (Fig. 1(A)) and characterized their structures as manghaslin (**1**), clitorin (**2**), rutin (**3**), and nicotiflorin (**4**) (Afzan et al., 2012; Lin and Harnly, 2007). These compounds were inactive when tested against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* (Table 1).

Anticipating that alkaloids detected in the active time window spectrum could possibly be the active constituents, and knowing that the alkaloid content in papaya leaves was low (Barger et al., 1937; Burdick, 1971; Bukhori et al., 2005; Greshoff, 1890), we prepared an alkaloidal fraction with the aid of an ion-exchange column. Piperidine alkaloids **5–9** (Fig. 2) were then separated by RP MPLC, and their structures were established by HR MS and NMR data.

Compound **5** was carpamic acid, a compound that had been previously reported as the degradation product formed upon hydrolysis of carpaine and pseudocarpaine (Barger et al., 1933; Govindachari et al., 1965; Vo et al., 1998). We confirmed its natural occurrence by LC–MS analysis of freshly prepared methanolic leaf extracts. Carpamic acid has also been synthetized from alanine

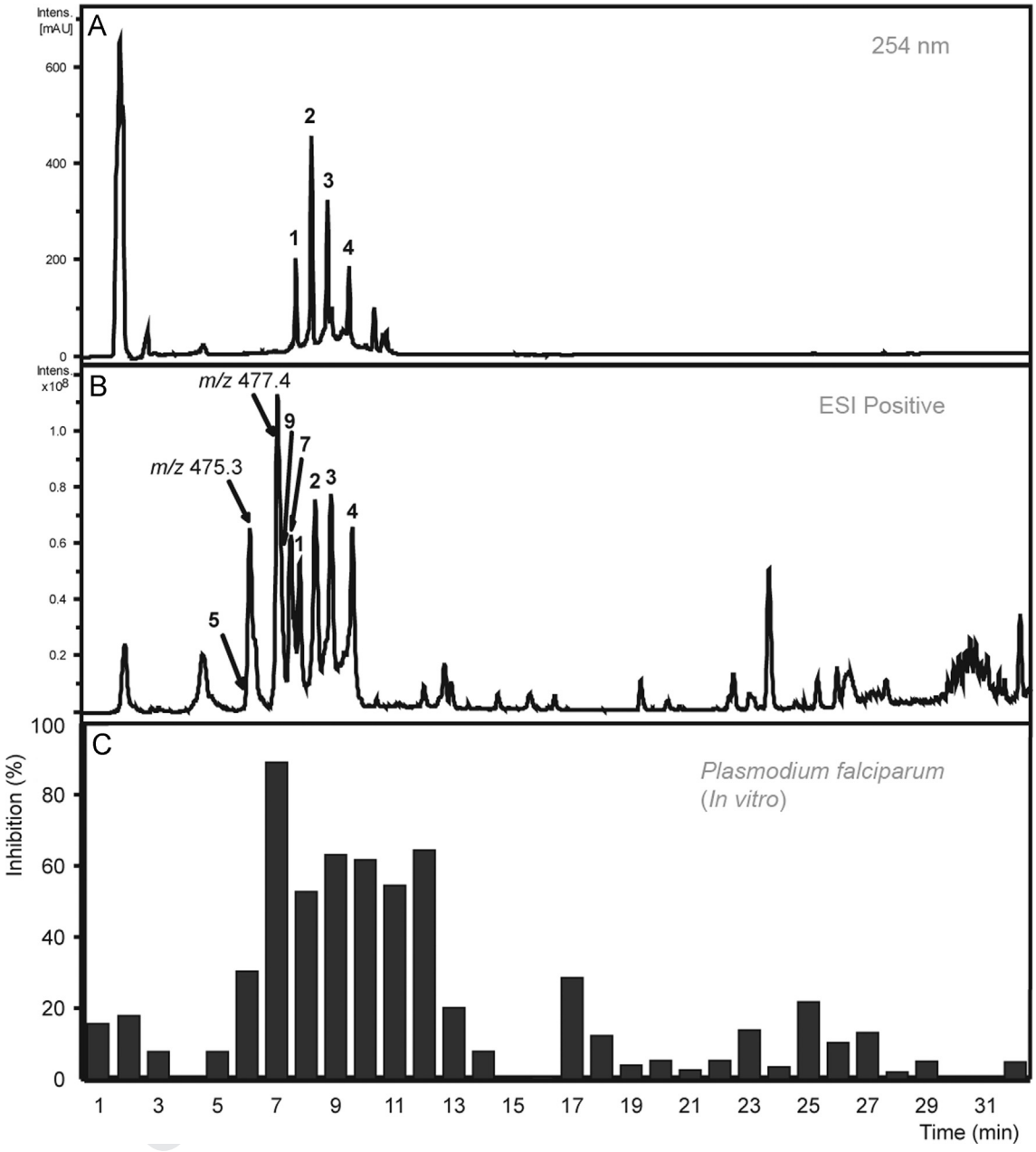

Fig. 1. HPLC-based activity profiling of a methanolic extract. UV chromatogram (A), LC–MS chromatogram (B), and activity profile (C).

Table 1
in vitro activity of compounds **1–9**.

Extract/compound	IC_{50} (μM)								
	Trypanosoma brucei rhodesiense	SI	*Trypanosoma cruzi*	SI	*Leishmania donovani*	SI	*Plasmodium falciparum*	SI	Cytotoxicity L6
1	73	> 1.6	n.d.	n.d.	n.d.	n.d.	> 13.2	> 9	> 119
2	63.5	> 1.9	n.d.	n.d.	n.d.	n.d.	> 13.5	> 9	> 121.6
3	73.2	> 2	n.d.	n.d.	n.d.	n.d.	> 16.4	> 9	> 147.4
4	51.8	> 2.9	n.d.	n.d.	n.d.	n.d.	> 16.8	> 9	> 151.5
5	119.4	3.2	n.d.	n.d.	n.d.	n.d.	> 194.4	< 1.9	379.9
6	138.6	> 2.7	n.d.	n.d.	n.d.	n.d.	> 77.1	4.8	> 368.7
7	12.7	1.7	16.3	1.3	> 209	< 0.1	0.2	107.5	21.5
8	35.8	1.2	27.8	1.6	> 195.9	< 0.2	1.8	24.2	43.5
9	41.1	0.7	30.1	0.9	> 209	< 0.1	1.0	28.2	28.2
Reference drugs	0.01		1.8		0.3		0.2		0.007

The IC_{50} values are expressed as mean value of two independent assay ($n=2$); Reference drugs: *Trypanosoma brucei rhodesiense* (melarsoprol), *Trypanosoma cruzi* (benznidasole), *Leishmania donovani* (miltefosine), *Plasmodium falciparum* (chloroquine), cytoxicity (podohyllotoxin); Selectivity Index (SI): quotient of IC_{50} in L-6 cells and IC_{50} against parasites; n.d.: not determined.

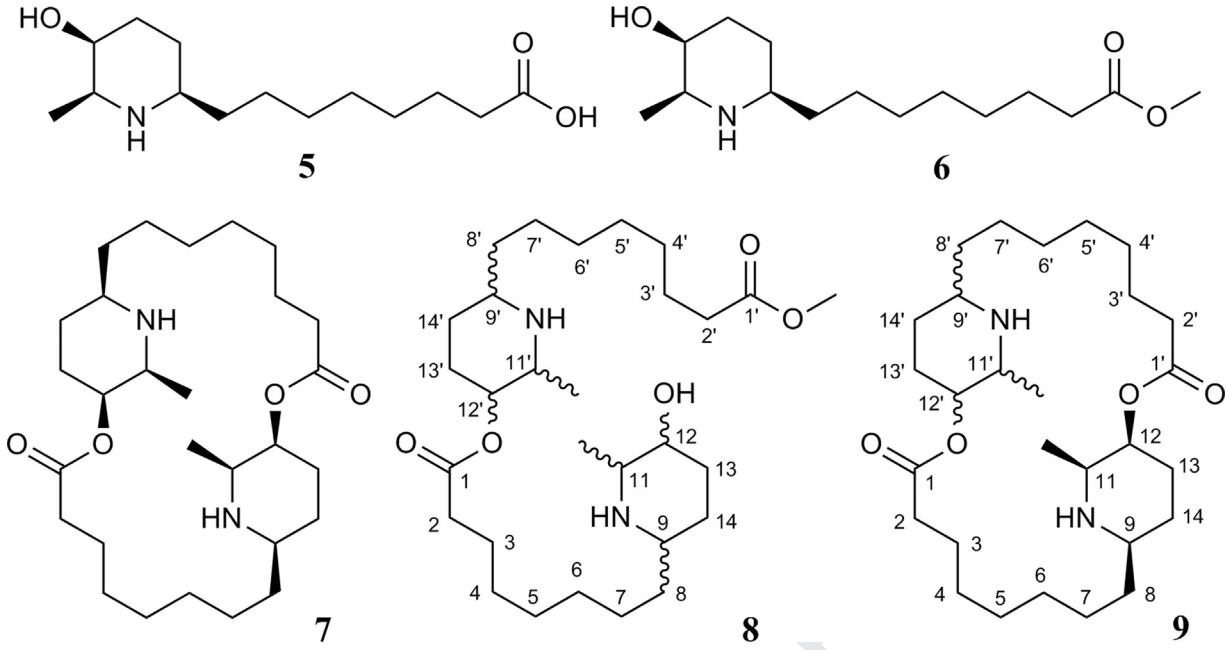

Fig. 2. Chemical structures of alkaloids **5**–**9**.

Q5 (Randl and Blechert, 2004; Masuda et al., 2006). Given that **5** lacked a chromophore, the absolute configuration could not be established *via* ECD. However, suitable crystals were obtained, and the absolute configuration of the piperidine ring was confirmed by X-ray diffraction as *2′R-5′S-6′S* (Fig. 3).

Methyl carpamate **6**, in contrast, was a likely artifact formed by methylation of carpamic acid, or by hydrolysis of carpaine during elution of the ion exchange column. The compound was detected by LC–MS in the alkaloidal fraction, but not in the methanolic extract. It has also been previously reported as a product of esterification of carpamic acid (Barger et al., 1933).

Chemical structure and absolute configuration of the major alkaloid, carpaine (**7**), have been previously reported (Govindachari et al., 1965; Sato et al., 2003). Since the ^{1}H and ^{13}C spectra showed severely overlapping signals in the high field region, and given that the chemical shift values slightly differed from reported data, we confirmed the structure with the aid of X-ray diffraction analysis and ECD spectroscopy. The X-ray structure of compound **7** (Fig. 3) was comparable to the one reported by Kabaleeswaran et al. (1999). The ECD spectrum of carpaine showed sequential positive and negative CEs at 240 and 215 nm, respectively. A calculated ECD spectrum was obtained by the time-dependent density functional theory (TDDFT) (Bringmann et al., 2008). The conformational search based on the X-ray data revealed 15 conformers within a 2 kcal/mol energy window from the particular global minimum. Conformational analysis using relative free energy showed high flexibility of the macrocyclic bislactone ring (Fig. 4). Calculation of the ECD spectra was performed by using TDDFT/B3LYP/6-31**, with MeCN as solvent (Supplementary material, Fig. S4). Experimental and calculated weighted ECD spectra were in good agreement. In particular, a diagnostic negative Cotton Effect (CE) at 210 nm (Fig. 5) was due to the $n \rightarrow \pi^*$ transition of the ester moiety. On the basis of these data the absolute configuration of **7** was confirmed.

Compound **8** was closely related to carpaine (**7**). The NMR data revealed that the opening of one lactone ring of carpaine formed a methyl-ester derivative (Fig. 2). Like other N-alkyl heterocycles (Lambert and Featherman, 1975) the NMR data of compound **8** showed broadened resonances which suggested slow conformational changes of the piperidine moiety. This precluded the assignment of the relative configuration with the aid of J coupling pattern analysis and NOESY experiments. The experimental ECD spectrum showed a positive CE around 200–220 nm contradictory to compounds **7** and **9** (Supplementary material Fig. S5). Taking into account all these considerations, we could not unambiguously

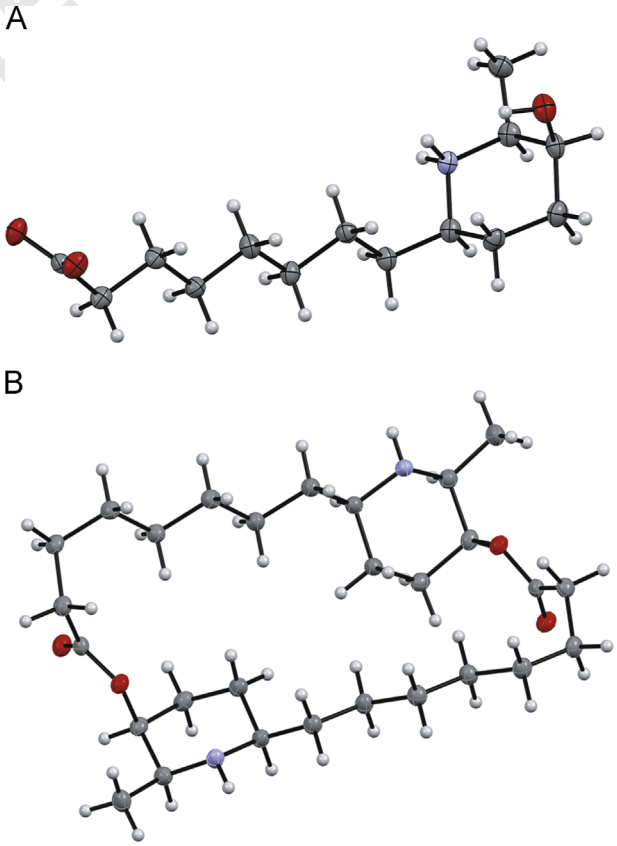

Fig. 3. X-ray structures of compounds **5** (A) and **7** (B).

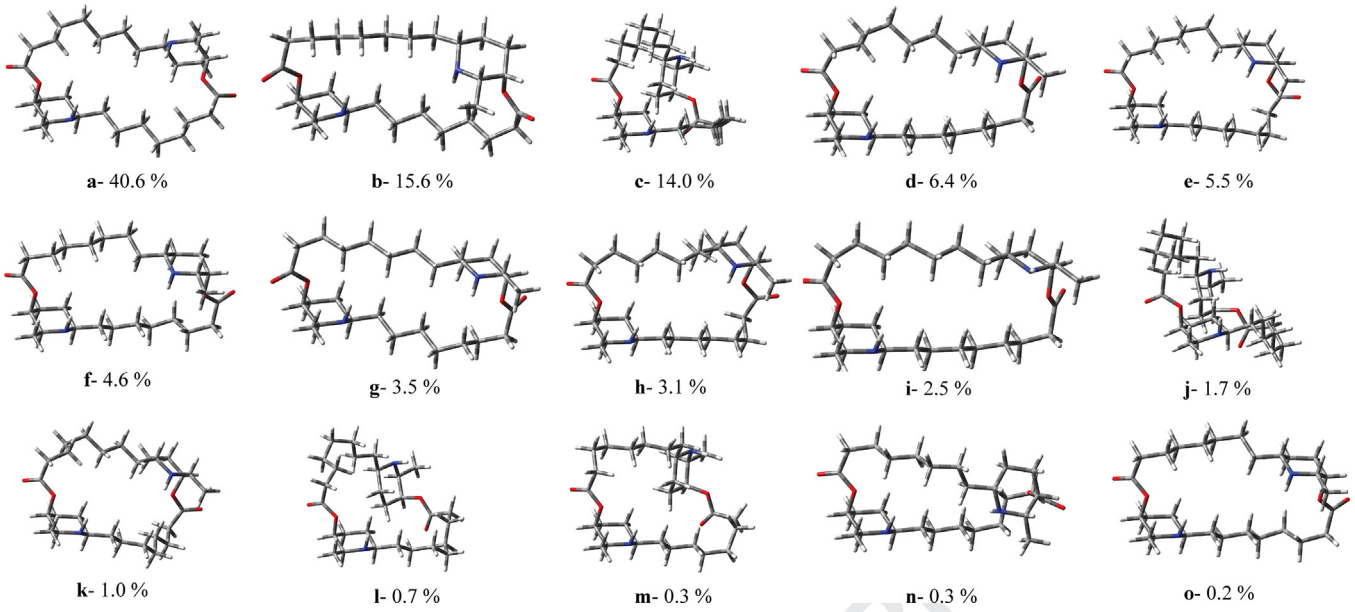

Fig. 4. Conformational analysis of compound **7**. Minimized conformers in the gas phase using DFT at the B3LYP/6-31G* level, within a 2 kcal/mol range from the global minimum.

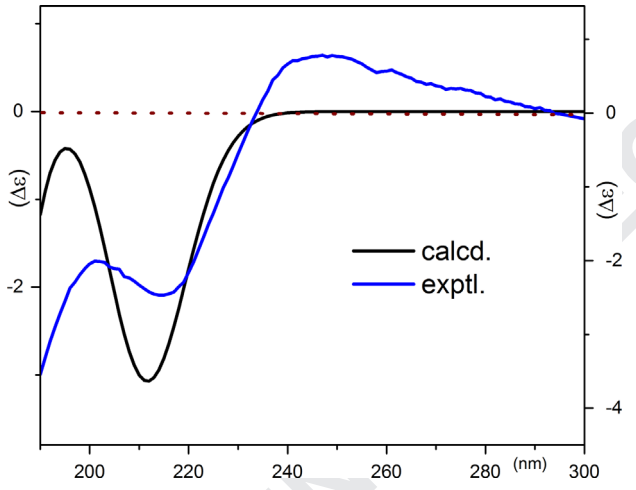

Fig. 5. Overlay of experimental (blue) and calculated (black) ECD spectra of **7**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assign the stereochemistry of compound **8**, but we assumed that it was an artifact formed through methanolysis of compound **7**.

An alkaloid named pseudocarpaine, possessing the same planar structure as carpaine (**7**) and compound **9**, had been previously found in trace amounts in papaya leaves by Govindachari et al. (1954), and later by Khuzhaev and Aripova (2000). However, the stereostructure of pseudocarpaine had not been established. We confirmed that alkaloid **9** was composed of two halves having the same gross structure as **7**, but differing in the stereochemistry of one piperidine ring. One of the two piperidine rings presented spectroscopic data similar to that of carpaine **7**, while the NMR resonances of the second ring differed significantly. Repeated attempts of crystallization failed, and we were not able to establish the 3D structure of compound **9**. We here report its ^{1}H and ^{13}C NMR shifts and ECD spectrum (see Supplementary material Fig. S5), since they may be helpful for future unambiguous identification of related dimeric alkaloids.

In the HPLC chromatogram of the MeOH extract (Fig. 1), we observed the presence of two additional compounds in the activity window, with masses of 475.3 and 477.4 that fitted with previously reported dehydrocarpaine II and I, respectively (Tang, 1979). Careful analysis showed that these two compounds were only present in the methanolic extract and not in the alkaloidal fraction. This suggested a possible degradation of these compounds during the alkaloidal enrichment procedure, and they could not be obtained in amounts sufficient for structure elucidation.

Alkaloids **5**–**9** were tested for *in vitro* antiprotozoal activity, and for cytotoxicity in L6 cells (Table 1). None of the compounds was active against *Leishmania donovani*, *Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*. In contrast, alkaloids **7**–**9** showed good activity against *Plasmodium falciparum*. The best compound in this series was carpaine (**7**), with an IC_{50} of 0.2 μM and a selectivity index of 107. The compound met the criteria for progression to *in vivo* testing. However, the *in vivo* test with mice using the 4-days suppress assay resulted only in a 11.9% reduction of parasitemia in mice, and the animals had to be euthanized.

Our findings of potent *in vitro* but lacking *in vivo* activity of carpaine are seemingly in contrast to the previously reported *in vivo* activity of an alkaloid containing papaya extract (Sulistyowati, 2000). In that study, a hydroalcoholic extract of papaya leaves was tested in the murine *Plasmodium berghei* model. Oral administration of the extract in doses of 13.3, 20, and 30 mg/10 g body weight per day led to a reduction of parasitemia by 54%, 64%, and 72%, respectively.

At this point, these contradictory results cannot be resolved. A possible explanation is that in a papaya extract accompanying constituents contribute to the activity, either *via* a pharmacokinetic interaction and/or pharmacodynamic synergy. Such cases have been reported for other antimalarial plants and compounds, such as *Artemisia annua* and quinoline alkaloids in *Cinchona* bark (Druilhe et al., 1988; Liu et al., 1989; Rasoanaivo et al., 2011).

4. Conclusions

Decoction from papaya leaves has been traditionally used in eastern Indonesia to prevent and treat malaria, and an ethanolic extract from papaya leaves had reportedly shown *in vivo* activity (Sulistyowati, 2000). HPLC-based activity profiling allowed an efficient tracking of the *in vitro* activity, and the active compounds were identified as piperidine alkaloids. However, when tested in

the murine *Plasmodium berghei* model, the major active compound **7** was not able to control parasitaemia.

Given the longstanding and wide use of papaya leaves as an anti-malarial remedy and its wide availability in malaria-afflicted regions, further investigations are warranted. Most importantly, the reasons for lacking *in vivo* activity of carpaine need to be investigated, and the role of other leaf constituents in the previously reported *in vivo* activity of extracts. Carpaine possesses good *in vitro* activity and selectivity, and therefore is a new scaffold for anti-plasmodial compound worth to be pursued.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.05.050.

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Supplementary Material

HPLC-based activity profiling for antiplasmodial activity in the traditional Indonesian medicinal plant *Carica papaya* L.

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Table 1. Bioactivity of extracts against *P. falciparum* (*in vitro*)

	Inhibition (%)	
Extract	4.81 μg/mL	0.81 μg/mL
Petroleum ether	12.7	4.1
EtOAc	61	8.7
MeOH	51.8	n.d

Inhibition was determined in triplicate; n.d.: not determined.

Table 2. Crystal data for carpamic acid **5**

Formula	$C_{14}H_{27}N_1O_3$
Formula weight	257.37
Z, calculated density	2, 1.190 Mg · m^{-3}
F(000)	284
Description and size of crystal	colorless plate, 0.030 · 0.140 · 0.230 mm^3
Absorption coefficient	0.657 mm^{-1}
Min/max transmission	0.91 / 0.98
Temperature	123K
Radiation(wavelength)	Cu K_α (λ = 1.54178 Å)
Crystal system, space group	triclinic, P 1
a	5.7032(3) Å
b	7.2035(5) Å
c	18.0105(11) Å
α	99.280(3)°
β	95.373(3)°
γ	97.835(3)°
V	718.35(8) $Å^3$
min/max Θ	2.503° / 67.976°
Number of collected reflections	12137

Number of independent reflections	4539 (merging r = 0.027)
Number of observed reflections	4400 (I>2.0σ(I))
Number of refined parameters	326
r	0.0386
rW	0.0423
Goodness of fit	1.0351

Table 3. Crystal data for carpaine **7**

Formula	$C_{28}H_{50}N_2O_4$
Formula weight	478.72
Z, calculated density	2, 1.105 Mg · m^{-3}
F(000)	528
Description and size of crystal	colorless block, 0.060 · 0.110 · 0.210 mm^3
Absorption coefficient	0.572 mm^{-1}
Min/max transmission	0.94 / 0.97
Temperature	100K
Radiation(wavelength)	Cu K_α (λ = 1.54178 Å)
Crystal system, space group	orthorhombic, P 2_1 2_1 2
a	14.3945(5) Å
b	18.3514(7) Å
c	5.4456(2) Å
α	90°
β	90°
γ	90°
V	1438.51(9) $Å^3$
min/max Θ	3.903° / 68.155°
Number of collected reflections	30121
Number of independent refections	2623 (merging r = 0.031)
Number of observed reflections	2608 (I>2.0σ(I))
Number of refined parameters	155
r	0.0230

rW	0.0254
Goodness of fit	1.1265

Fig. 1. ^{1}H-NMR spectrum of compound **7** ($CDCl_3$, 500.13 MHz).

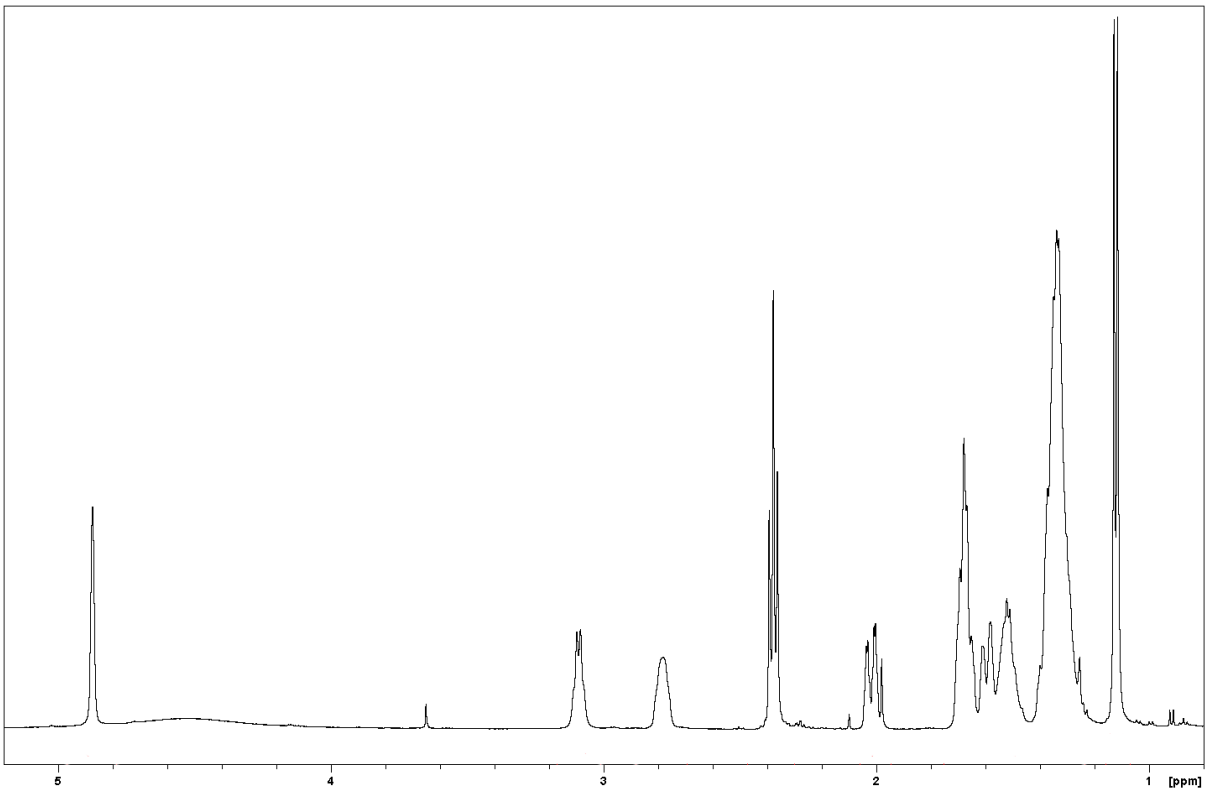


Fig. 2. ^{1}H-NMR spectrum of compound **8** ($CDCl_3$, 500.13 MHz).

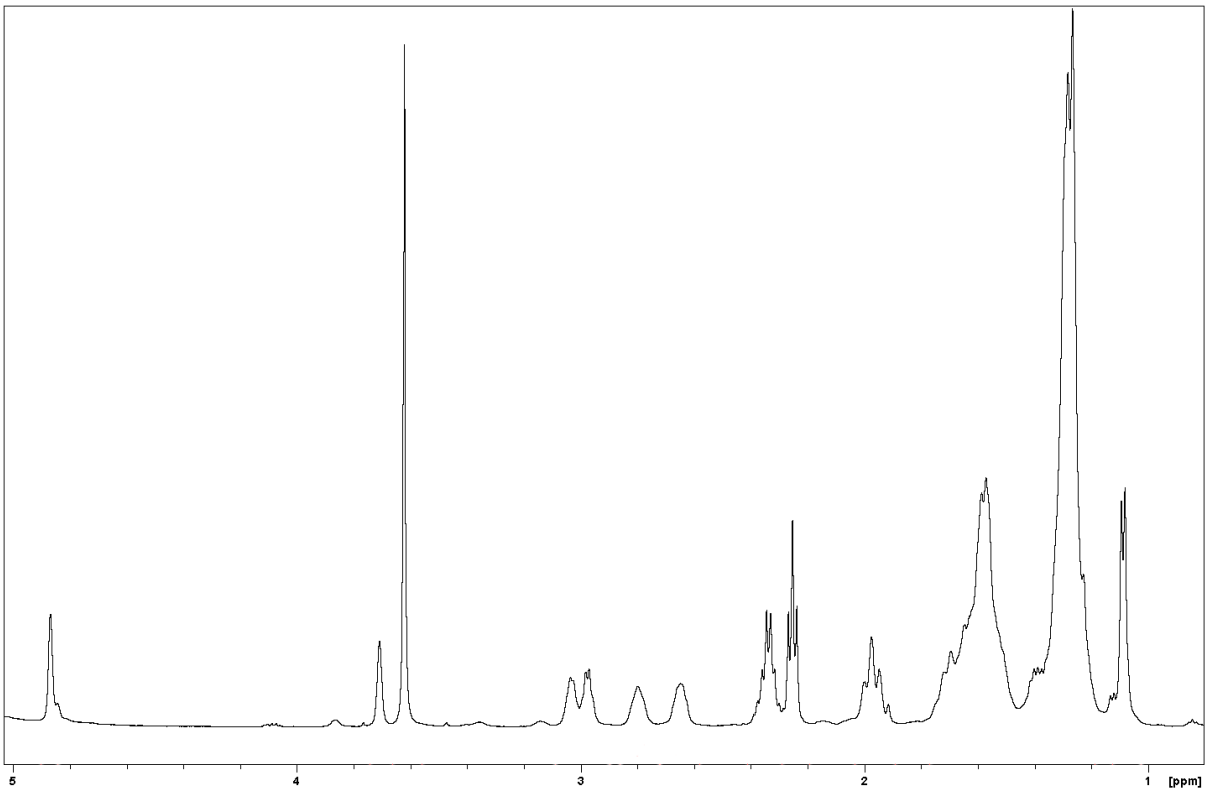


Fig. 3. ^{1}H-NMR spectrum of compound **9** ($CDCl_3$, 500.13 MHz).

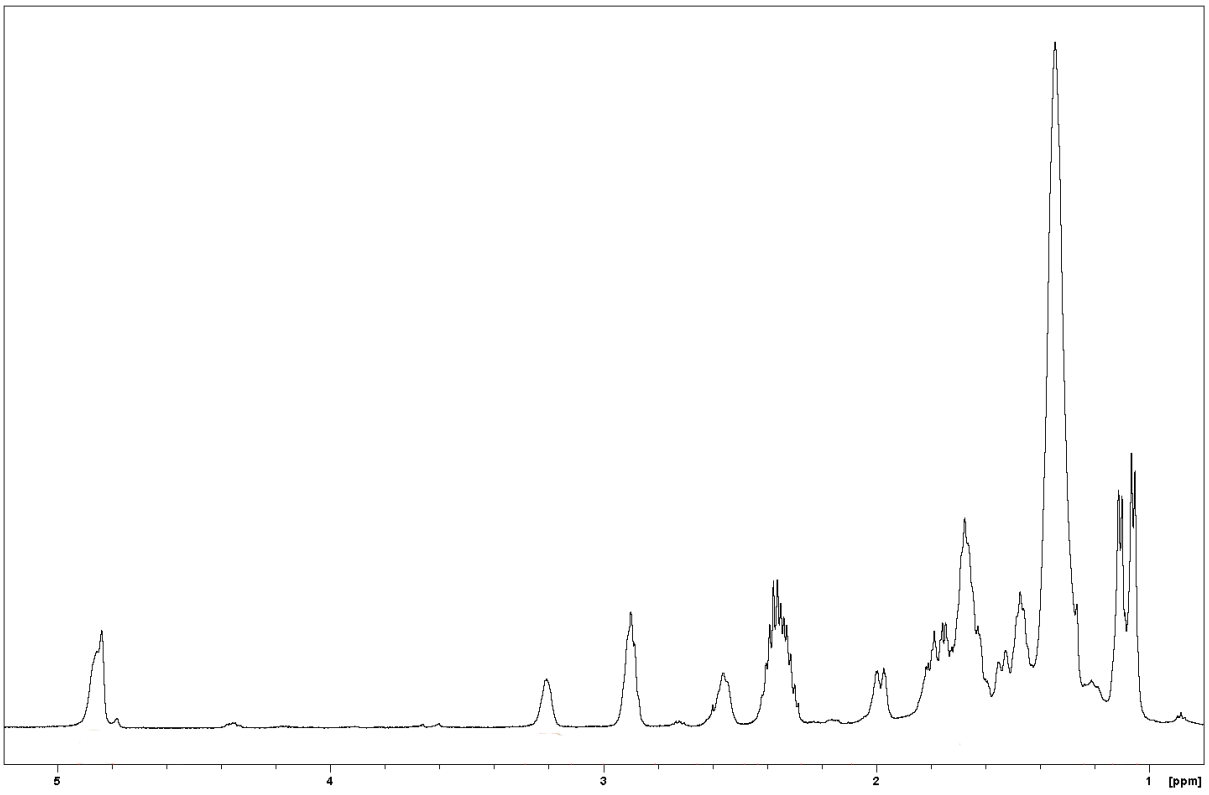


Fig. 4. Calculated ECD spectra of carpaine (7) conformers.

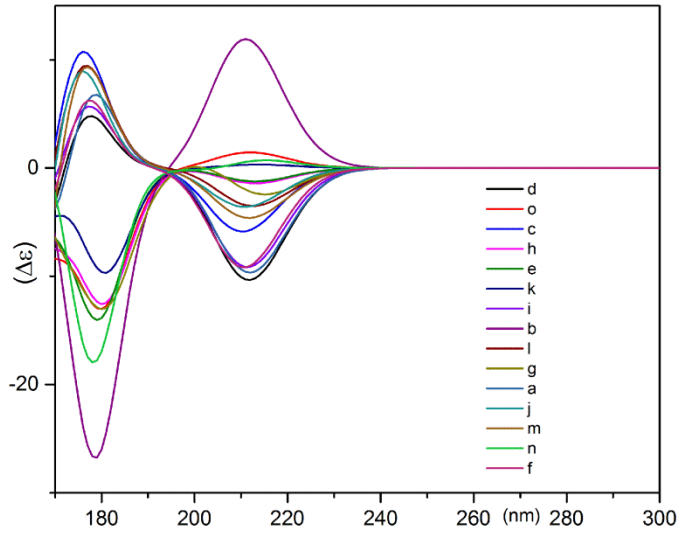
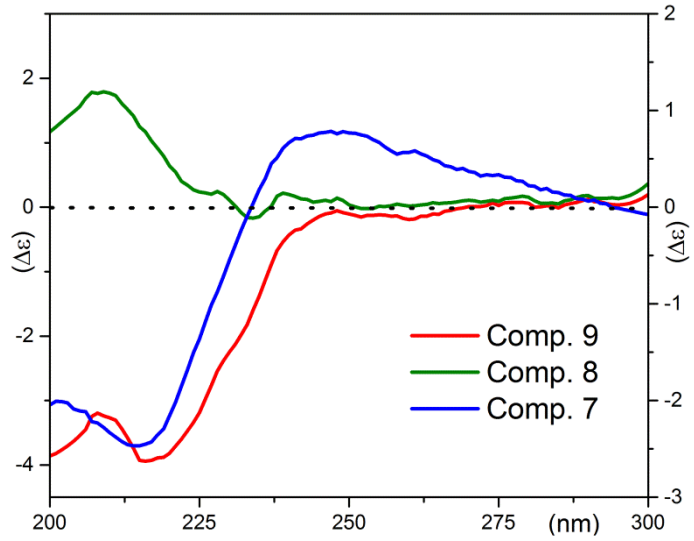


Fig. 5. Overlay of ECD spectra of compounds **7**-**9** in MeCN.



CHAPTER V

QUANTIFICATION OF THE ANTIPLASMODIAL ALKALOID CARPAINE IN PAPAYA LEAVES (*CARICA PAPAYA*)

Quantification of the antiplasmodial alkaloid carpaine in papaya leaves (*Carica papaya*)

Tasqiah Julianti, Mouhssin Oufir, Matthias Hamburger. *Planta Medica*, submitted.

Leaf samples from 28 places from Indonesia together with one sample from India were collected for the quantification of carpaine. Optimized pressurized liquid extraction was used to exhaustively extract carpaine in papaya leaves. UHPLC-MS/MS method was developed and partially validated to determine the amount of carpaine in the extract. The method was found to be valid and reliable for the intended application covering from nano to microgram amounts of compound. A wide carpaine concentration was observed from these 29 samples. No correlation of the carpaine content to the origin and leaf maturity was observed.

Optimization of the extraction method, recording and data analysis for the extraction method development, plant samples extraction, preparation of extract for quantification, UHPLC-MS/MS data interpretation and calculation of carpaine content, as well as draft writing and figure preparation are my contribution for this publication.

Tasqiah Julianti

Quantification of the antiplasmodial alkaloid carpaine in papaya leaves (*Carica papaya*)

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Abstract

Daily consumption of papaya leaves (*Carica papaya* L.) as greens and herbal infusion is common in some parts of Indonesia as a means for preventing malaria. Antiplasmodial activity of leaf extracts and of the main alkaloid carpaine were recently confirmed. A quantitative assay for determination of carpaine in papaya leaves was developed and validated. The assay involved extraction by PLE, and quantification with the aid of UHPLC-MS/MS. Extraction conditions were optimized with respect to solvent, temperature, and number of extraction cycles. The UHPLC-MS/MS assay was validated over a range of 20 – 5000 ng/mL (R^2 of 0.9908). A total of 29 papaya leaf samples were analyzed, and carpaine concentration in dry leaves was found to range from 0.02 to 0.31%. No obvious dependence on geographic origin and leaf maturity was observed.

Key words

carpaine, *Carica papaya*, Caricaceae, quantification, pressurized liquid extraction, UHPLC-MS/MS.

Abbreviations

UHPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectroscopy, PLE: pressurized liquid extraction, ASE: accelerated solvent extraction, DMSO: dimethyl sulfoxide, ULC: ultra liquid chromatography, ESI: electrospray ionization, EIC: extracted ion chromatogram, IPA: isopropyl alcohol, EtOAc: ethyl acetate, I.S.: internal standard, EMV: electron multiplier voltage, MRM: multiple reaction monitoring, SD: standard deviation, CV: coefficient of variation, RE: relative error, QC: quality control, LOD: limit of detection, LOQ: limit of quantification, ULOQ: upper limit of quantification, LLOQ: lower limit of quantification, S/N: ratio of signal to noise.

Introduction

Leaves from papaya trees (*Carica papaya* L., Caricaceae) are consumed as vegetable and tea in certain parts of Indonesia. The leaves are believed to benefit health by increasing appetite and breast milk production. Also, they are used as an anthelminthic, and as remedies for reducing fever, and for preventing and curing malaria [1–3]. In Eastern Indonesia (eg. Papua and Maluku islands) where malaria is still endemic, people consume papaya leaves as greens and/or tea on a daily basis to prevent the disease. The leaves are cooked in water before being eaten, and the remaining water may be served as tea. Tea can also be prepared from freshly ground leaves.

Papaya leaves contain various nutrients and minerals. Surprisingly little is known about the secondary metabolites in papaya leaves. The presence of flavonoids, saponins, tannins, anthraquinones, and cardiac glycosides has been reported, but these findings based on simple wet chemical assays are of very limited value [4–6]. Knowledge about alkaloids in papaya leaves is much more substantial. The piperidine alkaloid carpaine has been reported as the major alkaloid in leaves, and has also been found in the roots, barks, and seeds of papaya trees [7,8]. Other alkaloids in papaya leaves include dehydrocarpaine I and II, pseudocarpaine,and nicotine [8–10].

The antimalarial property of papaya leaf extract was confirmed in animal model [11]. We were able to corroborate this finding by *in vitro* testing of papaya extracts and, with the aid of HPLC-based activity profiling [12] we identified the alkaloid carpaine as the major antiplasmodial compound [13]. Carpaine possessed significant antiplasmodial activity *in vitro* (IC_{50} of 0.2 μM) and high selectivity towards the parasite.

In an attempt to further validate the traditional use of papaya leafs as an antimalarial, we wanted to determine the carpaine content in leaf samples from different locations in West Java. Also, we wanted to explore a possible influence of leaf age on carpaine concentration.

Given that so far no validated assay for carpaine had been published, we here report first on the development and validation of a protocol involving pressurized liquid extraction (PLE) and UHPLC-MS/MS. PLE has been shown to be highly suitable for quantitative extraction of plant material, and superior to conventional extraction techniques such as maceration or Soxhlet extraction [14–16]. Also, ASE was found easier to validate than other extraction methods [17,18]. Given that carpaine (**Fig. 1**) lacked a chromophore suitable for UV detection, an UHPLC-MS/MS assay was developed, whereby tandem mass spectroscopy was chosen for its superior sensitivity and specificity.

Results and Discussion

We first optimized conditions for a quantitative extraction of carpaine from papaya leave samples. Solvents of different polarities, extraction temperature, and number of extraction cycles were compared with respect to extraction yield and carpaine content.

When comparing solvents with increasing polarity (petroleum ether, CH_2Cl_2, EtOAc, and MeOH; five extraction cycles each), the highest yields of extract and carpaine were obtained with MeOH (**Fig. 3A** and **B**). The other three solvents resulted in lower extract yields, and incomplete extraction of carpaine. Given that carpaine is an alkaloid, we repeated extraction under alkaline conditions. It has been previously shown that moistening of powdered drug with dilute ammonia solution significantly increased extraction yield of alkaloids in PLE [15]. Under alkaline conditions the highest yield in carpaine was obtained with petroleum ether (**Fig. 3B**), while the yield of total extract (**Fig. 3A**) was lowest. This also resulted in cleaner MS chromatograms, and extraction with petroleum ether under mild alkaline conditions was therefore selected.

In PLE, higher extraction temperatures generally lead to higher extraction yields. However, thermal stability of analytes may be a limiting factor. We analyzed the carpaine yields obtained with extraction temperatures varying between 60 and 120°C (**Fig. 4**). The highest yield was found with a temperature of 90°C, while the slight decrease at higher

temperatures was likely due to thermal degradation. The above experiments were all carried out with five extraction cycles to ensure exhaustive extraction.

To optimize efficiency of the assay, we determined the minimum number of extraction cycles needed to achieve exhaustive (> 98%) extraction of carpaine. In an experiment with five consecutive extraction cycles, the relative yields were 79.6%, 14.6%, 4.63%, 1.03% and 0.12%, respectively (**Fig. 5**)**.** Hence, three extraction cycles were sufficient for the purpose.

Up to now, no quantitative assay for determination of carpaine has been developed. Earlier estimations of carpaine content were based on gravimetric assessment of crystallized alkaloid. More recently, qualitative methods using TLC, and UPLC-ESIMS methods for detection of carpaine were reported [10,19]. Therefore, we, developed and validated a quantitative UHPLC-MS/MS of carpaine.

As suitable internal standard (I.S.) we selected the alkaloid emetine. For calibration curves, two mass transitions were used for carpaine (qualifier and quantifier), and one transition for the I.S. (**Table 1**). Relative response was calculated by dividing analyte peak area to I.S. peak area. The parameters of the calibration curve (relative response = $A.x^2 + B.x + c$) were obtained by quadratic least square regression with a weighting factor of $1/x^2$. A typical calibration curve is given in supporting information. Limit of detection (LOD) and lower limit of quantification (LLOQ) were determined based on MS/MS response of a serial dilution of standard solution. LOD and LOQ were 1 ng/mL (S/N ≥ 3) and 10 ng/mL (S/N ≥ 10), respectively. However, because of the occurrence of some carry over, LLOQ was set at a concentration of 20 ng/mL. Since we observed some carry over, a more detailed carry-over assessment was needed. The assessment was carried out by injection of one blank solution (DMSO) directly after the measurement of a high analyte concentration sample or standard solution at the upper limit of quantification (ULOQ).

The average carry over was within the requirements of the FDA guidance, indicating that carry over has no impact on the measurements.

The assay imprecision was 2.85 – 4.17%, and inaccuracy was ±11.5%. The imprecision and inaccuracy of standard solutions (calibrators) were found as 0.87 – 12.9% and -4.08 – 1.92%, respectively, where LLOQ showed 0.874% CV and -0.141% RE. The average recovery rates obtained from two extracts from different sources spiked with standard solution spiked in amount of 47.5 ng and 237.5 ng were found as 97.3 and 102%, respectively. Considering the values of all validation parameters, the method was found to be precise and accurate for quantification of carpaine in papaya leaf extracts.

Prior to the quantitative analysis of carpaine in papaya leaf samples, we determined the dilution factor of extract solution that was needed to reach a final concentration that would be around the median concentration of the calibration curves. This was to ensure that a wide concentration range within the different samples was covered. A dilution factor of 40 times was found to be appropriate.

Indeed, analysis of 29 samples showed that the carpaine content in dry leaves varied between 0.02 – 0.31% (**Fig. 7**). Out of the 28 leaf samples from Indonesia, 15 were from older, and 13 were from younger leaves. However, when comparing the carpaine content in old and young leaves, we could not observe a clear relationship between age and alkaloid content. Overall, the highest carpaine content was observed in leaf samples from the Anyer area.

Previous studies on carpaine content in papaya leaves reported highly varying data, ranging from 0.011% to 3.7% [20 – 26]. In previous publications, carpaine was in most cases reported as the major alkaloid in papaya leaves, and our findings corroborate these earlier data. In contrast, dehydrocarpaine I and II were found to be more prominent than carpaine in leaves of Hawaiian origin [10], while choline has been reported as the major basic constituent in leaves of Nigerian origin [25].

Earlier studies on leaf age and alkaloid content gave conflicting results. In his publication reporting on the discovery of carpaine, Grishoff estimated the carpaine concentration in older leaves at 0.07%, and indicated that young leaves were likely to contain three to four

times higher concentration of the alkaloid [20]. In contrast, Barger *et al*. later emphasized that carpaine concentrations were increasing with age in the following order: seedling, young, and older leaves [21,22]. However, the same authors later found that the carpaine concentration was lower in older leaves than in seedlings [23].

In summary, we developed the first validated extraction procedure and quantitative assay for carpaine in papaya leaves. Our survey covering young and older papaya leaves of from different locations in Eastern Java revealed highly varying carpaine contents which were independent from leaf age.

Materials and Methods

Chemicals and reference compounds

Analytical grade solvents for extraction, and HPLC grade solvents were from Scharlau (Barcelona, Spain). Ammonium hydroxide (NH_4OH) (26%) was from Riedel-de Häen (Seelze, Germany). Formic acid (98 – 100%) was purchased from Sigma-Aldrich (Buchs, Switzerland), and Dimetyl Sulfoxide (DMSO) from Reuss Chemie (Tägerig, Switzerland). HPLC grade water was obtained by an EASYpure II (Barnstead, Dubuque, USA) water purification system. UPLC/MS grade acetonitrile (ACN), methanol (MeOH), formic acid, trifluoroacetic acid (TFA), acetone, and isopropyl alcohol (IPA) were from BioSolve (Valkenswaard, The Netherlands). Carpaine was previously isolated in our lab from papaya leaves. Purity of > 96 % was determined by HPLC-MS and NMR. Emetine dihydrochloride hydrate (100%) was from Sigma-Aldrich (MO, USA).

Plant Material

A total of 28 different leaf samples were collected in Western Java, Indonesia. The leaves were collected from small gardens around residential areas and small plantations, at six different locations shown in **Fig. 2**. Two locations were in the province of Banten: desa Cikoneng, kecamatan Anyer, Serang (S 6°4’40.026”, E 105°53’3.224”), and kelurahan Pamulang Barat, kecamatan Pamulang, Tangerang Selatan (S 6°20’20.526”, E 106°43’55.448”); two locations in the province of Jakarta: kelurahan Kedaung Kali Angke, kecamatan

Cengkareng, Jakarta Barat (S 6°9'1.058", E 106°44'58.365"), and kelurahan Srengseng Sawah, kecamatan Jagakarsa, Jakarta Selatan (S 6º20'17.1", E 106º49'57.0"); and two locations in the province of West-Java: desa Cimanggu, kecamatan Cibungbulang, Bogor (S 6°34'35.029", E 106°47'9.106"), and kampung Ciberem, kecamatan Cikarang Selatan, Bekasi (S 6°17'40.56", E 107°9'35.222"). Samples were collected from November to December 2011. Collected leaves were dried in the shadow for one week. One commercial leaf sample of Indian origin was purchased from Dixa AG (St. Gallen, Switzerland). Vouchers are deposited at the Institute of Pharmaceutical Biology, University of Basel.

The leaves were collected in a way representing different stages of leaf maturity: old leaves were collected from the lower part of the tree, and young leaves were collected from the top. Old and young leaves were either from the same tree, or from adjacent trees of the same location.

Optimization of extraction method

Leaf samples were extracted by pressurized liquid extraction (PLE) using an ASE 200 extractor with solvent module (Dionex; Sunnyvale, CA, USA). For extraction, 1.0 g of ground leaves were filled into a 22-mL extraction cartridge. Five consecutive extraction cycles were performed, using the following standard settings: preheat time 1 min, heating time 5 min, static extraction 5 min, flush 100% solvent of cell volume, purge 120 sec with nitrogen. The pressure was set at 120 bar. The following parameters were optimized: extraction solvent, extraction temperature, and number of extraction cycles. In addition, the effect of moisturizing the leaf powder prior to extraction with diluted NH_4OH solution (33%, w/v) was evaluated.

Optimization of carpaine extraction was monitored by HPLC-MS, using an Agilent HPLC series 1100 system consisting of degasser, binary high pressure mixing pump, column oven, PDA detector (Waldbronn, Germany), coupled to an Esquire 3000 Plus ion trap MS (Bruker Daltonics, Bremen, Germany). MS spectra were recorded in ESI positive ion mode for the range of m/z 100 – 1000 Hystar 3.2 software (Bruker Daltonics) was used for controlling the

LC_MS system, and for data analysis. Separations were performed with an Atlantis® dC18 HPLC column (4.6 x 150 mm, 5 μm particle size; Waters, Wexford, Ireland).

Development and validation of UHPLC-MS/MS method

For quantitative analysis a 1290 Infinity LC system coupled to a 6430 triple quadrupole mass spectrometer with ESI interface was used. Data were processed with MassHunter Workstation software version B.06.00 (all Agilent; Waldbronn, Germany). The 1290 Infinity LC system consisted of a binary capillary pump G4220A, an autosampler G4226A, a cooling system G1330B, a thermostatted column compartment G1316C, and a FlexCube G4227A. Separation was performed at 55˚C on a Kinetex XB-C18 (100 x 2.1 mm i.d., 1.7 μm particle size; Phenomenex; Torrance, CA). The mobile phase consisted of 0.1% formic acid in H_2O (solvent A), and 0.1% formic acid in ACN (solvent B). The following gradient profile was used: 2% B for 1 min, linear gradient to 25% B in 6 min, and 100% B for 1, and back to equilibrium condition of 2% B for 2 min. The flow rate was 0.4 mL/min. Sample injection volume was 1 μL, and the autosampler was set at 25˚C. Needle wash solution consisted of MeOH/ACN/IPA/H_2O (1:1:1:1, v/v) and the injector needle was washed for 20 sec. FlexCube was set at a flow rate of 1 mL/min for 10 sec in order to reduce the carry over.

MS parameters were automatically set, and then optimized manually. The following final settings were used: N_2 drying gas temperature 300˚C, at a flow rate of 6 L/min; nebulizer pressure of 30 psi; capillary voltage of 4 kV; delta EMV 0 V. Quantification was performed using multiple reaction monitoring (MRM) in the positive ionization mode. MRM transitions of carpaine and internal standard (I.S.) are given in Table 2, and representative MRM traces are shown in **Fig. 6**.

An analytical run consisted of two sets of seven calibration samples (calibrators), six quality control samples (QCs), two calibrator zero (blank spiked only with internal standard), four blanks (DMSO), and analyte samples.

Calibration curves. Standard solutions were prepared by a serial dilution of carpaine stock solution of 10 μg/mL in DMSO. Calibration curves were obtained with carpaine solutions of

20, 100, 250, 500, 1000, 2500, and 5000 ng/mL in DMSO containing 2000 ng of emetine (I.S.). Emetine working solution was prepared freshly for every analytical run. Two sets of series dilutions were prepared and measured at the beginning and end of the analytical run.

Limit of detection, lower limit of quantification, and carry-over. A serial dilution of standard solution was used for determination of limit of detection LOD and lower limit of quantification LLOQ. Carry over was determined by injection of blank (DMSO), after injection of the upper limit of quantification ULOQ (calibrator 7).

Quality controls. Quality control samples (QCs) were prepared in a similar procedure to standard solution in three defined concentrations: QC low at 60 ng/mL (corresponding to 3 x LLOQ), QC medium at 2500 ng/mL (corresponding to mid-range), and QC high at 4000 ng/mL (corresponding to 80% of ULOQ). These QCs were injected randomly in duplicate between the real samples.

Recovery. Accuracy was determined by recovery rates of carpaine spiked in two defined amounts (7.5 and 237.5 ng) to two different extracts.

Carpaine assay in extracts

Extracts were prepared by PLE. Solvent was removed with rotary evaporator, and the dry extracts were stored in glass vials at -20°C until analysis. Sample preparation for analysis was carried out as follows: to the dry extracts 6 mL of MeOH was added, sonicated for 3 min, and centrifuged. The supernatant was transferred into a 25 mL volumetric flask, and the residue was extracted for three additional times as above. The combined extracts were made up to 25.0 mL with MeOH in the volumetric flask. An aliquot of 1 mL of this solution was sampled, evaporated under N_2 flow, redissolved with 1 mL of DMSO to obtain the sample stock solution. Three working solutions were prepared by dilution of stock solution by factor of 40 in DMSO containing emetine. The final sample solution for analysis contained I.S emetine at 2000 ng/mL. The concentration of carpaine in the extract was calculated statistically and reported as percentage to the dry weight of the leaves.

Supporting Information

The calibration curves of standard solution of first calibration set, and quality control sample data and analysis are available as supporting information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Chemical structure of carpaine and emetine (I.S.).

Fig. 2. Sites for sampling of papaya leaves in Western Java, Indonesia.

Fig. 3. Extraction yields (A), and relative yields (B) of carpaine determined by LC-MS (n = 2). PE: petroleum ether; DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol.

Fig. 4. Influence of extraction temperature on carpaine yield.

Fig. 5. Optimization of number of extraction cycles. Relative amounts of carpaine extracted in cycles 1 to 5, and total yield.

Fig. 6. MRM chromatograms of carpaine (t_R 6.54 min; quantifier and qualifier), and emetine ((t_R 4.43 min).

Fig. 7. Carpaine content in papaya leaves. Samples from different locations are given in different colours. Content was determined in triplicate, and error bars indicate SD. † younger leaves. ‡ unknown leaf maturity. Plain: old leaves.

Table

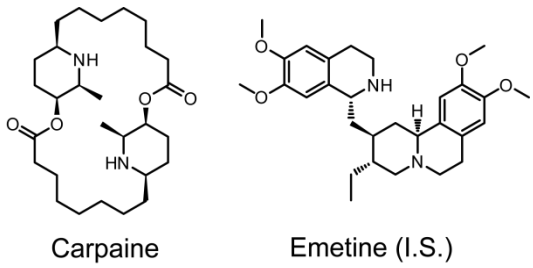
Table 1. MRM Parameters for Quantitative Analysis (ESI Positive Ionization)

Compound	Retention time (min)	Precursor ion (*m*/*z*)	Fragmentor (V)	Product ion (*m*/*z*)	Collision energy (eV)
Emetine (I.S.)	4.43	481.3 $[M+H]^+$	217	246.2	34
Carpaine	6.43	479.39 $[M+H]^+$	202	240.2* 222.2**	34 42

*Quantifier (highest response); ** Qualifier

Figures

Fig. 1



Carpaine Emetine (I.S.)

Fig. 2

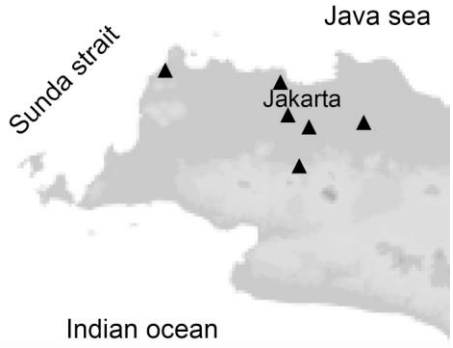


Fig. 3

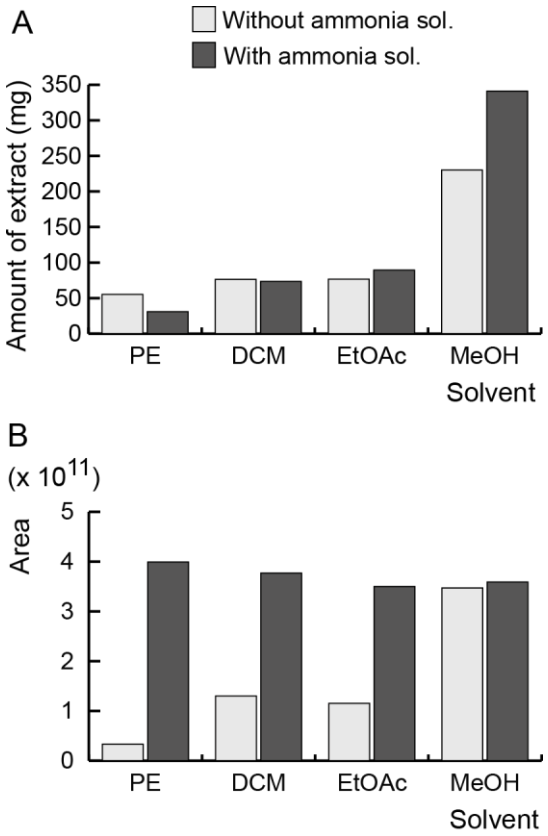

A
Without ammonia sol.
With ammonia sol.
Amount of extract (mg)
350
300
250
200
150
100
50
0
PE
DCM
EtOAc
MeOH
Solvent
B
(x 10^11)
Area
5
4
3
2
1
0
PE
DCM
EtOAc
MeOH
Solvent

Fig. 4

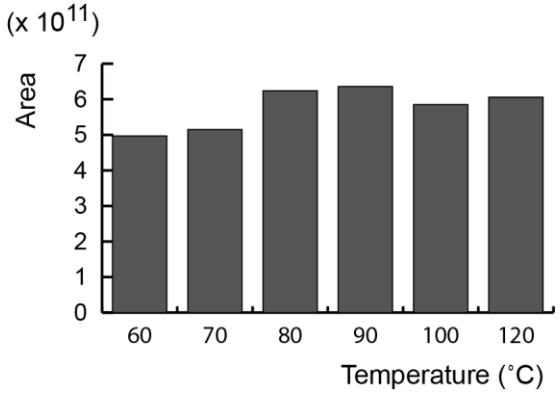

(x 10^11)
Area
7
6
5
4
3
2
1
0
60
70
80
90
100
120
Temperature (°C)

Fig. 5

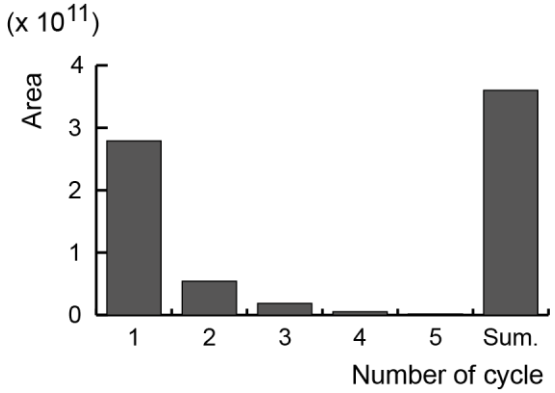


Fig. 6

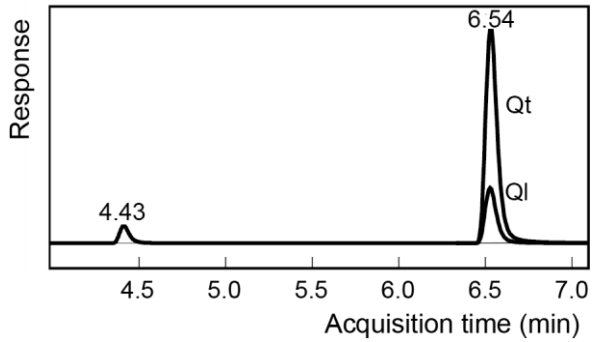
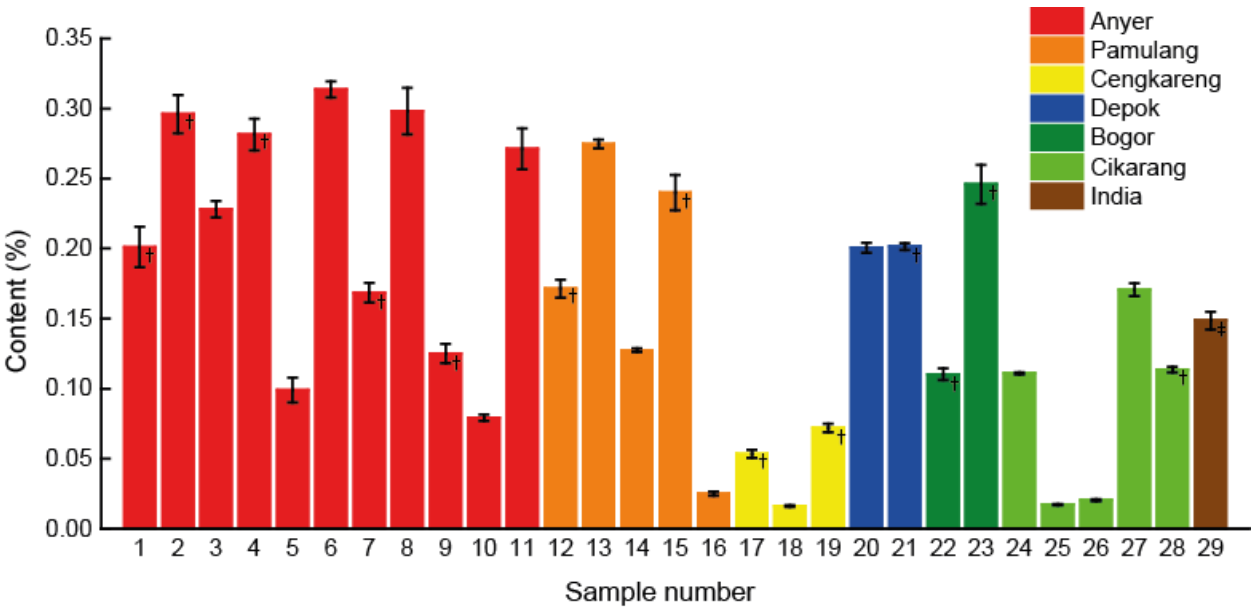


Fig. 7



Supporting Information

Quantification of the antiplasmodial alkaloid carpaine in papaya leaves (*Carica papaya*)

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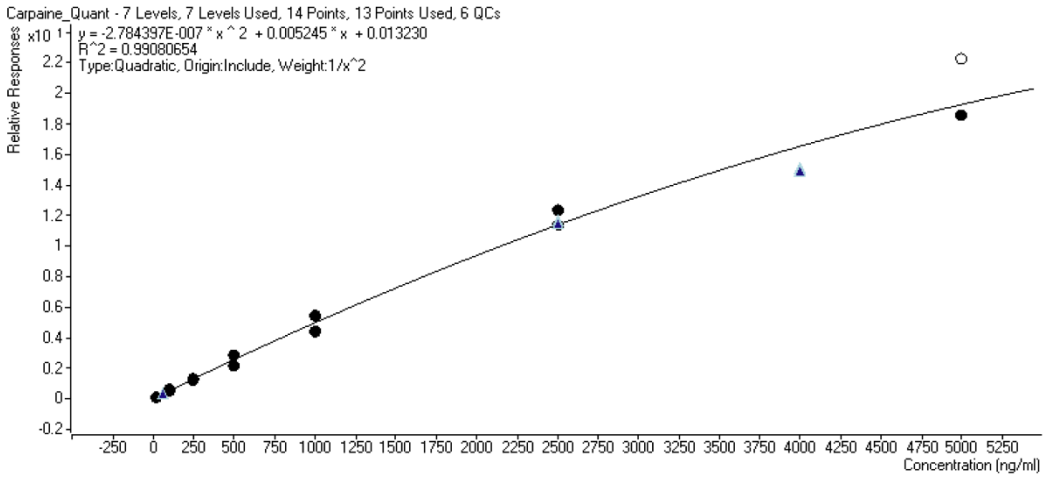
Calibration curves of standard solution of first calibration set. Calibrators are signed as dots and QCs are signed as triangles.



Quality control sample data and analysis.

	QCL 60 ng/mL	QCM 2500 ng/mL	QCH 4000 ng/mL
1	68.0	2570	3501
	63.7	2519	3541
2	68.0	2400	3677
	68.1	2379	3438
Mean	66.9	2459	3539
S.D.	2.17	102	101
CV %	3.25	4.17	2.85
RE %	11.5	-1.62	-11.5

CHAPTER VI

CONCLUSION & OUTLOOK

With this study, the use of medicinal plants has proven to be valuable in the search for natural antiprotozoals active against *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*. Discovery of the active principles in the active extracts was achieved with HPLC-based activity profiling (Potterat and Hamburger, 2013) using only microgram amount of extracts. This approach is evidently effective and fast in identifying the active principles for the extracts of the plants. Isolation of structurally related constituents was useful for preliminary structure activity relationship analysis.

Saussurea costus roots are known for malaria in the Indian traditional medicine system. From the screening of the extracts of our in-house library, the ethyl acetate extract of this plant showed good antitrypanosomal activity *in vitro*. With HPLC-based activity profiling, the activity of the two active constituents, costunolide and dehydrocostuslactone, was distinctly shown amongst the other compounds in the extract. A simple structure activity relationship was proposed with additional two inactive compounds and three other compounds from different sources. Compounds bearing a germacranolide skeleton displayed higher activity than those with guaianolide and eudesmanolide scaffolds.

In Indonesia, *Carica papaya* leaf is traditionally used for prevention and treatment of malaria. Previous studies on the extract reported antiplasmodial activity *in vitro* and *in vivo*. HPLC-based activity profiling of a methanolic extract of papaya leaves showed an activity window that correlated with a complex pattern of peaks in the LC-MS chromatogram. Analysis of the UV and mass spectra indicated the presence of flavonoids and alkaloids. Chromatographic isolation delivered four flavonol glycosides, two monomeric piperidine alkaloids, and three dimeric alkaloids. Dimeric alkaloids showed the highest antiplasmodial activity, followed by flavonols and monomeric alkaloids. The major alkaloid, carpaine, showed high activity and remarkable selectivity. A further *in vivo* investigation in mice with carpaine, however, led only to a weak reduction of parasitemia. Additional investigations on carpaine content in leaf samples from various origins in Indonesia revealed a wide range of concentrations. For this particular case, the use of HPLC-based activity profiling together with early identification and dereplication of active compounds in the extract with sensitive MS detection were very helpful. The antimalarial activity of this plant had been reported in the literature, however, the active principles had not been identified. 2D NMR experiments

including COSY, HSQC, HMBC and NOESY led us to distinguish between two isobaric compounds, carpaine and a stereoisomer. The configuration of these two compounds could not be assessed with NOESY due to heavily overlapping signals. However, the absolute configuration of carpaine was confirmed via X-ray crystallography.

The discrepancies of *in vivo* activity between the extract and isolated compounds are common in natural products research. One general explanation for this is the overall synergistic effect of the numerous compounds present in the extract. The influence of the flavonoids for their activity on the parasite *P. falciparum* has been comprehensively proposed through different mechanisms (Ferreira et al., 2010). A direct effect of flavonol glycosides on *P. falciparum* K1 strain was also observed in this study. However, with the currently limited data, further studies are required.

The compounds isolated from *Saussurea costus* (Asteraceae) and *Carica papaya* (Caricaceae) bear sesquiterpene lactone and piperidine alkaloid skeletons, respectively. Numerous sesquiterpene lactones have been reported as to possess antiplasmodial and antitrypanosomal activity. In contrast, dimeric piperidine alkaloids represent a new scaffold for antiplasmodial compounds.

The tropical rainforests of Indonesia offer numerous plants that have been traditionally used as medicines. However, with the current state of natural product research in the country, the potential of this biodiversity is largely unexplored. The establishment of an extract library for high-throughput screening, together with the application of HPLC-based activity profiling could significantly accelerate the discovery of new bioactive molecules. These active molecules could serve many purposes, such as new medicines by themselves, new templates for derivatives and inspiration for synthetic drugs. Since the use of traditional medicines is widely accepted in the Indonesian medical system, the discovery of the active principles in traditionally used plants would be very useful for extract standardization, which in turn would help to increase the safety and efficacy of the traditional medicine dosage forms.

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