Lansium domesticum: skin and leaf extracts of this fruit tree interrupt the lifecycle of Plasmodium falciparum, and are active towards a chloroquine-resistant strain of the parasite (T9) in vitro

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Abstract

Malaria remains a global problem in the light of chloroquine-resistant strains of Plasmodium falciparum. New compounds are needed for the development of novel antimalarial drugs. Seed, leaf, and fruit skin extracts of Lansium domesticum, a common fruit tree in South-East Asia, are used by indigenous tribes in Sabah, Malaysia for treating malaria. The skin and aqueous leaf extracts of the tree were found to reduce parasite populations of the drug sensitive strain (3D7) and the chloroquine-resistant strain (T9) of P. falciparum equally well. The skin extracts were also found to interrupt the lifecycle of the parasite. The data reported here indicate that extracts of L. domesticum are a potential source for compounds with activity towards chloroquine-resistant strains of P. falciparum.

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1. Introduction

Malaria is a global public health problem that primarily affects the people of the subtropical and tropical zones of the world. However, travelers to these endemic malaria zones are also at risk of contracting the disease. Global estimates of malaria deaths range from 0.7 to 2.7 million per year with over 75% of deaths occurring among children younger than 5 years in tropical Africa (Breman, 2001).

In humans, the disease is caused by four species of the malaria parasite (Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, and Plasmodium ovale) of which P. falciparum is the most virulent and potentially deadly. Falciparum malaria is found throughout the tropics although infection in Africa tends to be endemic with high transmission rates while in South America, the Indian subcontinent, South-East Asia and China, transmission rates tend to be lower and seasonal in nature (Winstanley, 2000). The other three species are distributed unevenly throughout the tropical (and in the case of P. vivax, in some temperate) regions and do not generally cause severe disease although they are often linked with anemia and low birth weights. In addition, P. vivax and P. ovale have liver forms which can cause relapses if left untreated, and P. malariae can persist in the blood for years if not eradicated completely (Gilles, 1993).

Efforts to eradicate malaria began in the 1950s under the auspices of the WHO and were initially successful, but by the 1970s, the disease was resurgent again (Onori et al., 1993). Today, the eradication of malaria is one of the most pressing global public health issues (Winstanley, 2001). The reasons for the resurgence of malaria are complex and beyond the scope of this paper but one reason is the paucity of new antimalarial drugs that are both cheap and effective against drug-resistant parasite strains.

1.1. Drug resistance

Many of the antimalarial drugs in widespread use today are losing their effectiveness in the face of multidrug-resistant strains of Plasmodium that are becoming increasingly prevalent around the world (Hyde, 2002; Winstanley et al., 2002; Wongsrichanalai et al., 2002). Quinine, derived from the bark of the cinchona tree, was first isolated in 1820 by Pelletier and Caventou (see Foley and Tilley, 1998 for historical review of the quinoline antimalarials) and is still used today for cases of severe chloroquine (CQ)-resistant malaria in Africa. The drug is not suitable for widespread...
use in third world countries due to its cost, dosing regime and toxicity, however, it remains useful as a second or third line drug in Africa but less so in South-East Asia due to widespread resistance in the region (Winstanley, 2001; Wongsrichanalai et al., 2002).

CQ was first introduced as an antimalarial drug in 1945 due to its potency, low cost, and ease of use but by 1957, resistance to CQ in Thailand was already being reported (Foley and Tilley, 1998). Today, there is widespread resistance to the drug in South-East Asia and in most parts of Africa although it is still widely used for economic reasons (Winstanley, 2001). The remaining cost-effective alternative to CQ is a combination of two drugs—sulfadoxine and pyrimethamine (PS), also known as Fansidar. Unfortunately, resistance to PS occurs very quickly, and resistance to PS is already well established in South-East Asia and in parts of Africa. Eight countries in Africa have switched to PS as the first line drug against malaria and already failure rates of 20–45% and 60% in first and second clinical treatment, respectively, are already seen, and the drug is expected to become ineffective by 2006–2007 (Winstanley, 2001; Winstanley et al., 2002).

The Chinese have used teas made from Artemisia annua L. (sweet or annual wormwood) to treat fevers and malaria for over 2000 years. The active component present in these teas is artemisinin, a highly potent compound that can reduce parasite loads within 48 h and has a short half life of 13 h when taken orally (van Agtmael et al., 1999). Artemisinins and its derivatives are also effective against drug-resistant malaria (Meshnick et al., 1996; Balint, 2001). The short half life of the artemisinins, however, leads to recrudescence rates of 44–54% for 3-day dosing regimes, and this led to the suggestion that the artemisinins be used in combination with drugs with a long half life to obtain increased cure rates and to slow down the growth of drug resistance to the adjuvant drug (White and Olliaro, 1996; van Agtmael et al., 1999). The results of a long-term study on combination treatment with artesunate and mefloquine have borne this out indicating that the artemisinins may perhaps best be used in combination with other drugs such as mefloquine (Nosten et al., 2000). Unfortunately, mefloquine is too expensive for widespread use and such combinations would therefore be of little practical use in third world countries.

1.2. Developing new antimalarial drugs

The development of new, potent, and cheap antimalarials should be a global priority today if malaria is to be controlled in the near future. With the exception of the artemisinins and new combinations of existing drugs, there are few new antimalarial drugs being developed that are both cost-effective and have potent activity towards the drug-resistant variants of the parasite (Winstanley et al., 2002). The identification of natural product preparations used against malaria in traditional medicine is also likely to lead to compounds or classes of compounds that can be developed as new antimalmodials (Kirby, 1996, 1997). The chances of finding new drugs through investigation of natural products are good given the results of the past—about 40% of all drugs approved between 1983 and 1994 were natural products or derived from natural products and on average, 70% of new antibacterial/anticancer drugs were derived solely from natural products (Cracq et al., 1997). The structural diversity afforded by natural products is also greater than that obtained from most combinatorial processes based on heterocyclic compounds which increases the chances of finding a compound with activity (Harvey, 1999).

Lansium domesticum Corr., is a genus of small trees from the family Meliaceae found wild and cultivated in Malaysia and surrounding countries in the region (Thailand, Vietnam, Philippines, and Indonesia). The fruit is known locally as langsat, duku, duku-langsat, or dokong. The tree grows to a height of 40–50 ft with long leaves that are pinnate, dark green, and glossy on the surface. The flowers are found in clusters on the trunk and the older branches of the tree and tend to be small, fleshy, white-yellow, and mostly bisexual. The fruit grows in clusters and is small and round with a diameter between 3 and 5 cm, with a yellow leathery skin that can be thick or thin depending on the variety. The flesh of the fruit is juicy and translucent, and comes in five or six segments with seeds contained in at least 1–3 segments (Morison, 1987). The fruit is produced commercially although it is not unusual for a household to have a langsat tree on their property. The fruit can be acidic or sweet depending on the variety and local growing conditions, and is generally eaten directly after peeling. The dried peel is also burnt as a mosquito repellent in parts of Java, and an arrow poison has reportedly been made from the fruit peel and bark of the tree (Morison, 1987). A survey carried out to determine the antimalarial measures taken by the indigenous lowland tribes of Sabah (North Borneo) indicated that the seeds, leaves, and bark of Lansium were used in the absence of standard antimalarial drugs (Cos-Singh, unpublished data). As a result, we decided to examine the antimalarial activity of L. domesticum extracts in vitro to determine if these extracts had activity against the CQ-resistant strain of P. falciparum, T9.

2. Methodology

2.1. Extraction of L. domesticum

The seeds, leaves, and fruit skin of L. domesticum were washed, air dried, and pulverized before extraction with solvents. Each plant part was extracted sequentially with diethyl ether (Mallinkrodt), reagent grade methanol (R&M), and Ultrapure water (Elga Water Systems). Plant material (300–350 g) was immersed in ether and left for 48 h at room temperature, and the resulting solutions concentrated in vacuo to obtain a dark yellow oil (typical yields were in...
the range 1–2.5%, w/w). The ether extracted material was air dried before extraction with the next solvent. A Soxhlet apparatus was used for the methanol extraction; in a typical extraction, 350 g of plant material was extracted continuously with methanol for 6–8 h. The resulting solutions were then concentrated to an oil in vacuo. Typical yields were in the range 3–4 g (0.9–1.1%, w/w). Aqueous extracts of the seed, skin, and leaves were obtained by incubation with methanol, and the suspension stirred for 6–8 h with gentle heating (<80 °C). The resulting dark yellow solutions were frozen and lyophilized (Iishin, FD5525, Korea) to yield brown, bygroscopic powders. Typical yields obtained were between 2 and 3 g (0.7–1%, w/w). Methanol and ether extracts were stored at −20 °C while the water extracts were stored in a dry box until required. A clean white powder was precipitated from ether skin extracts with methanol, stored in a dry box until required. A clean white powder was precipitated from ether skin extracts with methanol, and this was used in assays instead of the original crude ether extract.

Stock solutions (1 mg/ml) of the water and methanol extracts were made in media and sterile filtered prior to use. However, the ether extracts were only sparingly soluble in aqueous media, and solutions of the extracts in DMSO precipitated when diluted in media. Saturated solutions of the ether extracts in media (nominally 1 mg/ml) were therefore prepared, centrifuged, and filtered to remove insoluble particles before dilution. The concentrations of the ether extracts reported in this work are, therefore, not known exactly.

2.2. In vitro culture of P. falciparum

P. falciparum was cultured in vitro according to methods established previously (Trager and Jensen, 1976). Briefly, parasites were grown in erythrocytes suspended in RPMI 1640 medium (Gibco-BRL) supplemented with human serum (10%), HEPES (25 mmol), and gentamicin (40 μg/ml; Gibco-BRL). The blood cultures were maintained at 37 °C in candle jar conditions and 10% haematocrit levels. Thin blood smears were prepared, fixed with methanol, stained with Giemsa (10% in PBS), and examined by microscopy daily to monitor parasitic populations. Culture media was changed daily and fresh blood was added to the wells to dilute parasite populations as required. Excess parasite cultures were frozen in a glycerol/mannitol solution (28% glycerol, 3% mannitol, w/v in 0.9% NaCl solution) and stored at liquid nitrogen vapor temperatures for future use.

2.3. Parasite synchronization

When parasite cultures were established, the parasite’s lifecycle was synchronized as described previously (Lambros and Vanderberg, 1979). Infected red blood cells were washed with RPMI 1640 media and suspended in an aqueous solution of d-sorbitol (Sigma, 5% w/v). Under these conditions, red blood cells containing mature trophozoites and schizonts were lysed, effectively killing the parasite within. Only erythrocytes infected with ring forms or young trophozoites survived the treatment. The parasite cultures were synchronized 2–3 times over several lifecycles. Thin blood smears were obtained daily, and the parasite forms examined for complete synchronization (>90% of one particular life cycle stage). Synchronized parasite cultures were used for all assays described in this work.

2.4. In vitro assays

The antiparasitical effects of the extracts were determined using a modification of the in vitro microtechnique described previously (Rieckmann et al., 1978; Ang et al., 1995). Briefly, infected erythrocytes (50 μl, 0.2% parasitemia) suspended in media supplemented with gentamicin and human serum (20%) were added to test solutions (50 μl) in a 96 well microplate: parasitemia (%)P and haematocrit in final solutions were 0.1 and 10%, respectively. Test solutions consisted of extract dissolved in RPMI 1640 media and sterilized by filtration. Final extract concentrations in the well were 400 μg/ml with the exception of extract SKE where the concentration was unknown (see Section 2.1). Control solutions containing no extract or CQ (20 nmol) were also included as part of the assay. The parasites were then incubated in test solutions at 37 °C under candle jar conditions for 48 h. Thin blood smears were made and stained with Giemsa. The degree of parasitemia was determined by examining a minimum of 2000 erythrocytes for infection by parasites using microscopy. The parasitemia was determined from the number of parasites in 2000 erythrocytes, normalized to control cultures.

2.5. Life cycle effects

In order to determine the effect of the extracts on the parasite’s life cycle, the ratio of rings, trophozoites and schizonts in T9 cultures exposed to extracts were determined, and the results compared with those from control cultures containing media or CQ (30 nmol). Extract concentrations of 1 mg/ml were used except in the case of ether extracts where a saturated solution was used. The experiments were carried out in 12 well plates with higher %P (4%) and larger solution volumes (1 ml) than in the in vitro assays but otherwise the procedure remained the same. Thin blood smears were taken, fixed, and stained with Giemsa every 24 h for 3 days. Lifecycle stages in parasite cultures were examined, and their ratios calculated daily based on examination of >100 infected erythrocytes per slide. The ratio of rings to trophozoites and schizonts in the presence of media, CQ (30 nmol), SKW, and SKE, are shown in Fig. 2a–d, respectively. The %P is shown as a line above the bars. The purpose of this experiment was to examine changes in the life cycle stages so the initial parasitemia was higher than that used to determine extract activity (4 vs. 0.1%P, respectively). Thus
From the seeds, leaves, and fruit skins of L. domesticum and Community Medicine. Nine extracts were obtained and leaves have been deposited in the Institute of Health, a local land owner. Dried samples of the fruit skin, seed, and surfactants from local markets, and the leaves obtained from a tree belonging to a local land owner. Dried samples of the fruit skin, seed, and leaves have been deposited in the Institute of Health and Community Medicine. Nine extracts were obtained from the seeds, leaves, and fruit skins of L. domesticum and tested for antimalarial activity (data not shown). This preliminary assay identified four extracts (leaf–methanol, leaf–water, skin–water and skin–ether; LEM, LEW, SKW, and SKE, respectively) that reduced parasitaemia more than 50%. The four extracts were therefore studied in greater detail, and the results shown in Fig. 1.

3. Results and discussion

3.1. Extract activity

The fruits of L. domesticum were purchased from local markets, and the leaves obtained from a tree belonging to a local land owner. Dried samples of the fruit skin, seed, and leaves have been deposited in the Institute of Health and Community Medicine. Nine extracts were obtained from the seeds, leaves, and fruit skins of L. domesticum and tested for antimalarial activity (data not shown). This preliminary assay identified four extracts (leaf–methanol, leaf–water, skin–water and skin–ether; LEM, LEW, SKW, and SKE, respectively) that reduced parasitaemia more than 50%. The four extracts were therefore studied in greater detail, and the results shown in Fig. 1.

The difference in response between the two strains of P. falciparum, 3D7 and T9 to CQ is evident. 3D7 parasite populations are lowered by approximately 50% more than T9 parasite populations, reflecting the intrinsic resistance of T9 parasites to CQ. The data for CQ was included to show the difference in response between 3D7 and T9 to CQ. Unfortunately no comparison can be drawn between this potent antimalarial and the extracts reported since the former is a pure compound while the latter are crude mixtures. At concentrations of 400 μg/ml (see Fig. 1), extract SKW was most effective against both strains of P. falciparum (3D7 and T9). Parasite response to LEW was similar but the extract was slightly less effective than SKW. Antimalarial effects were also observed with extract LEM although the T9 strain was less sensitive to the extract than 3D7. The concentration of SKE is unknown (see Section 2.1) but is considerably less than 400 μg/ml. However, its activity towards both parasite strains appears to be just as effective as the other extracts.

3.2. Effect of the skin extracts on the parasite's life cycle

The effect of SKE on the parasite’s life cycle was first observed during the in vitro assays; parasite cultures that had been exposed to the skin extracts appeared to have a high number of tight rings compared to control cultures and cultures exposed to leaf and seed extracts. Therefore an attempt to quantify this observation was made using T9 cultures as described in Section 2.5. The results of this experiment are shown in Fig. 2, and are quite startling for extract SKE, less so for SKW. Parasites were synchronized and experiments were started when >90% of the parasites were in the young trophozoite form. The parasite populations cultured in media alone and CQ solutions have similar distributions of blood stage life forms reflecting the normal development of the parasite over time. The slight dip in parasite population after 48 h and subsequent recovery in the CQ solutions reflects the resistance of the T9 strain to CQ, and is not unexpected. The population profiles of cultures exposed to both skin extracts are however quite different. The parasite population falls steadily while the percentage of rings increases with time. In the presence of SKW, the change is gradual, taking up to 72 h before the ratio of rings reaches 80%; however, in the presence of SKE the change is much more dramatic—almost 90% of the parasite life forms are present as rings after 24 h exposure to SKE. These tight rings were not viable despite replacement of the extract solution with fresh media and dilution with fresh erythrocytes. The parasite population in control and CQ solutions treated this way rebound whereas the parasites exposed to skin extracts remained mordant. At present, there is no explanation for why the parasites become such tight rings following exposure to the extracts. Presumably this is one result of the extract’s active component(s) but until the active ingredient is isolated and studied, no conclusions concerning its mode of action.
Fig. 2. Changes in the lifecycle of the parasite (T9) with time. The bars indicate the percentage of rings, trophozoites, and schizonts (black, blank, and striped bars, respectively) found in each culture with time. The change in %P (normalized to control wells) with time is indicated in the line above the bars. Control cultures contain media without extract and 30 nmol CQ (a and b, respectively). Test solutions contain SKW (1 mg/ml) and SKE (unknown concentrations; c and d, respectively).

action can be made. The results, however general, are encouraging as they show compounds present in the fruit skin of L. domesticum are as active towards CQ-resistant strains of P. falciparum as to drug sensitive strains. Although the data presented here are preliminary, it is clear that the leaves and fruit skin of L. domesticum contain compounds that, either singly or in combination, have antimalarial properties against the CQ-resistant strain of P. falciparum, T9.

The current work in our laboratories is focused on fractionation of the extracts and isolating groups of compounds/pure compounds to determine the active component responsible for the extracts’ antimalarial activity. Recently, we reported the isolation of a cyclic amino acid (\(n\)-methyl-4-hydroxyproline from methanolic extracts of the fruit skin (Yapp et al., 2002) although the compound itself only exhibited antimalarial activity at concentrations greater than 1 mg/ml. There are few other reports on compounds isolated from L. domesticum and none on its use for the treatment of malaria. Nishizawa et al. (1988, 1989) have reported the isolation of three tetranotriterpenoids from the seed (1988), and new cycloartanoid triterpene compounds from the leaves of the plant (1989). The latter compounds also showed inhibitory effects on skin tumor promoters in vitro but none have been tested for antimalarial activity in vitro.

4. Conclusion

This paper is, to the best of our knowledge, the first to verify that L. domesticum has antimalarial activity, and was initiated by reports on the use of L. domesticum to combat episodic fevers and malaria by indigenous peoples of Sabah, Malaysia. The work presented here is a good example of how indigenous knowledge of plants with medicinal qualities can lead to potentially useful compounds. Our data show that the extracts of the leaves and skin exhibit activity towards the P. falciparum, and of particular interest, are active towards a CQ-resistant strain of the parasite, T9.

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References


