

## *In vitro* antimalarial activity of extracts of some plants from a biological reserve in Costa Rica

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Received 27-V-2011. Corrected 20-IX-2011. Accepted 21-X-2011.

**Abstract:** Treatment with the usual antimalarial drugs, have induced parasite resistance, reinforcing the need to finding natural antimalarial components that would be found on plants from the forest. Therefore, we decided to look for these components in Costa Rican plants from a protected forest area. Fresh and dry extracts of roots, bark, leaves, flowers and fruits of 25 plants from a biological reserve in Costa Rica, Reserva Biológica Alberto Manuel Brenes (REBAMB), were studied *in vitro* for the presence of substances with antimalarial activity. By studying the inhibition of *P. berghei* schizogony, we assessed the antimalarial activity of several plant extracts: *Aphelandra aurantiaca*, *A. tridentata* (Acanthaceae); *Xanthosoma undipes* (Araceae); *Iriartea deltoidea* (Arecaceae); *Neurolaena lobata* (Asteraceae); *Senna papillosa*, *Pterocarpus hayessi*, *Lonchocarpus pentaphyllus* (Fabaceae); *Nectandra membranacea*, *Persea povedae*, *Cinamomum chavarrianum* (Lauraceae); *Hampea appendiculata* (Malvaceae); *Ruagea glabra*, *Guarea glabra* (Meliaceae); *Psidium guajava* (Myrtaceae); *Bocconia frutescens* (Papaveraceae); *Piper friedrichsthali* (Piperaceae); *Clematis dioica* (Ranunculaceae); *Prunus annularis* (Rosaceae); *Siparuna thecaphora* (Siparunaceae); *Solanum arboreum*, *Witheringia solanacea* (Solanaceae); *Ticodendrum incognitum* (Ticodendraceae); *Heliocarpus appendiculatus* (Tiliaceae) and *Myriocarpa longipes* (Urticaceae). We used different parts of the plants as well as fresh and dried extracts for testing IC50. The solid content of the extracts ranged from 1-71.9µg/mL. The fresh extracts showed stronger activity than the dry ones. Since the plants showing the strongest antimalarial activity are very common in Central America, and some similar genera of these plants have shown positives results in South America, we considered important to present these findings for discussion. On the other hand, this is the first systematic study of this kind ever realized in a circumscribed and protected area of Costa Rica. Rev. Biol. Trop. 60 (2): 881-891. Epub 2012 June 01.

**Key words:** malaria, antimalarial plants, *in vitro* antimalarial activity, Costa Rican plants, *Plasmodium berghei*.

Malaria is a very important tropical disease, with a reported human morbidity approaching 300-500 million worldwide and a mortality of 1-2 million deaths per year (WHO 2005, Wrigth 2005). *Plasmodium falciparum*, the most pathogenic representative of this species, is responsible for the great majority of cases (Tramputz *et al.* 2003, Batista *et al.* 2009, Kakkilaya 2008). Confirming this fact, the

geographical areas where this species is more frequent, such as Africa, also have the greater incidence of deaths due to malaria infection.

From 1973-1983, Costa Rica implemented strong antimalarial programs, that virtually eradicated the disease and the incidence became near zero for many years (Vargas 2001). The epidemiological control was neglected for the same reason and new infection outbreaks

appeared again, as a consequence of disordered immigrations and immune deficiencies in segments of the population (AIDS). The outbreaks are not as important as they were in 1991-2000, but are certainly increasing with time (Trejos *et al.* 2010).

Unfortunately, continuous and frequent treatments with antimalarial drugs, such as chloroquine, have induced parasite resistance (Baird 2004), reinforcing the need for finding more natural antimalarial components. We believe that the best candidates for that would be found on plants from the forest.

Besides, the World Health Organization (WHO), aiming to reduce parasite resistance and to find natural products for this purpose, has promoted research in this area (Simpson 2002, Anthony *et al.* 2005). Quinine was the first natural drug found for malaria treatment, but because of its high toxicity, its use is limited to only some very qualified cases (WHO 2010). For *P. falciparum* resistant strains, the alternative treatment actually is the use of artemisinin derivatives from plants of the genus *Artemisia*. (Kakkilaya 2008, WHO 2010). Research has continued in laboratories of many developed as well as undeveloped countries (Saxena *et al.* 2003). Several studies regarding this matter have made important contributions to this subject in the past in Costa Rica (Castro *et al.* 1996, Chinchilla *et al.* 1998, 2001, 2003).

While, in Africa and Asia there have been many studies that have reported active substances against *P. falciparum* (Pillay *et al.* 2008, Soh & Benoit-Vical 2007). In Latin-America most of the studies came from South America (Krettli *et al.* 2001, Botsaris 2007, Valadeu *et al.* 2009 among others) and very few from Central America (Kohler *et al.* 2002, Franssen *et al.* 1997); we believe that our study represents a new contribution to this scientific area.

WHO and OPS (Organización Panamericana de la Salud) recommend, as an official model for research with antimalarial components, the use of *Plasmodium berghei* strains (Ramesar *et al.* 2008).

Based on the previous knowledge, we decided to perform a more integral study of

Costa Rican plants for antimalarial activity. We choose to work in a more protected and less disturbed forest area called "Reserva Biológica Alberto Manuel Brenes" (REBAMB).

This study have three fundamental objectives: 1) Reveal the natural diversity present in REBAMB, 2) Demonstrate the importance of our biodiversity in ethnobotany, especially concerning the treatment of important diseases, such as malaria, and finally, 3) disseminate our studies, in an attempt to help the discovery of new drugs that could improve human health.

## MATERIAL AND METHODS

**Plants:** A total of 25 different plants (Table 1) were collected from October 2007-December 2008 in the biological reserve, previously mentioned (REBAMB), owned by the University of Costa Rica. This reserve is located 42km Northeast from San Ramón, (10°13'49" N - 84°36'10" W), Alajuela, Costa Rica, with an altitude that varies from 600-1 640 meters above sea level, and an average temperature of 21°C, a relative humidity of 98% and rainfall of 3 461mm per year, thus representing a variety of climates and ecological niches (Sánchez 2000).

Plants were selected according to previous works (Sittenfeld *et al.* 1999, Chinchilla *et al.* 2008) that showed that extracts obtained from insects feeding from certain plants, had antimalarial activity. Identification and selection of those host families and species was definitive in choosing the plants for this study.

All the plants were identified by an expert botanist, who based his findings on previous publications (Gómez-Laurito & Ortíz 2004, Barrantes 2004), the geographical location was established with the help of a GPS. All the fresh material collected, was carefully labeled and stored at our herbarium in UCIMED. A photographic register and phenologic analysis was performed for all plants directly in the field.

In all cases, the samples obtained consisted of bark, roots, young and mature leaves, as well as flowers and ripe or unripe fruits. All these materials were placed in individual

TABLE 1  
Families and species of plants from the REBAMB with antimalarial activity

Family	Species	Actividad*
Acanthaceae	<i>Aphelandra aurantiaca</i> (Scheidw.) Lindl. Laura Martínez 18.	Inactive
Acanthaceae	<i>Aphelandra tridentata</i> Hemsl. Laura Martínez 19	Active
Araceae	<i>Xanthosoma undipes</i> (K. Koch & C.D. Bouché) K. Koch Laura Martínez 15.	Inactive
Areceaceae	<i>Iriartea deltoidea</i> Ruiz & Pav. Laura Martínez 16	Inactive
Asteraceae	<i>Neurolaena lobata</i> (L.) Cass. Laura Martínez 23	Very active
Fabaceae	<i>Senna papillosa</i> (Britton & Rose) H.S. Irwin & Barneby Laura Martínez 33.	Active
Fabaceae	<i>Pterocarpus hayesii</i> Hemsl. Laura Martínez 12.	Inactive
Fabaceae	<i>Lonchocarpus pentaphyllus</i> (Poir.) Kunth ex DC. Laura Martínez 24.	Inactive
Lauraceae	<i>Nectandra membranacea</i> (Sw.) Griseb. Laura Martínez 7.	Very active
Lauraceae	<i>Persea povedae</i> W.C. Burger Laura Martínez 14.	Active
Lauraceae	<i>Cinnamomum chavarrianum</i> (Hammel) Kosterm. Laura Martínez 28.	Active
Malvaceae	<i>Hampea appendiculata</i> (Donn. Sm.) Standl. Laura Martínez 1.	Very active
Meliaceae	<i>Ruarea glabra</i> Triana & Planch. Laura Martínez 4.	Active
Meliaceae	<i>Guarea glabra</i> Vahl Laura Martínez 26.	Inactive
Myrtaceae	<i>Psidium guajava</i> L. Laura Martínez 26.	Very active
Papaveraceae	<i>Bocconia frutescens</i> L. Laura Martínez 2	Very active
Piperaceae	<i>Piper friedrichsthali</i> C. DC. Laura Martínez 13.	Inactive
Ranunculaceae	<i>Clematis dioica</i> L. Laura Martínez 11.	Active
Rosaceae	<i>Prunus annularis</i> Koehne. : Laura Martínez 10	Inactive
Siparunaceae	<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC. Laura Martínez 38	Very active
Solanaceae	<i>Solanum arboreum</i> Dunal Laura Martínez 22	Active
Solanaceae	<i>Witheringia solanacea</i> L'Hér. Laura Martínez 30.	Very active
Ticodendraceae	<i>Ticodendron incognitum</i> Gómez-Laur. & L.D. Gómez Laura Martínez 8.	Active
Tiliaceae	<i>Heliocarpus appendiculatus</i> Turcz. Laura Martínez 3.	Inactive
Urticaceae	<i>Myriocarpa longipes</i> Liebm. Laura Martínez 9.	Inactive

\*Very active: less than 5 µg/mL, Active: >5 to 50µg/mL, Weakly active: >50 to 100 µg/mL, Inactive: >100µg/mL.

plastic bags and transported in a cold container to the laboratory.

Each part of the plant was further divided into two portions: one was kept as fresh material, while the other was dried for subsequent analysis.

**Preparation of extracts:** The fresh material coming from all parts of the plants was carefully washed and then finely chopped; the other portion was dried and prepared as a coarse powder in order to ease extraction. For screening purposes, we used 15g of fresh or 10g of dried material. They were placed in separate 250mL amber bottles, and then extracted

with 100mL of 70% ethanol for a week at room temperature, with occasional agitation. These extracts, were vacuum filtered through a Buchner funnel using Whatman 1 paper and then concentrated at 40°C with a rotary evaporator (Buchi R-114); this procedure completely eliminated ethanol.

#### ***In vitro* antimalarial assay:**

- Parasite strain: A *P. bergheis* strain (NK 65) obtained from American Type Culture Collection (ATCC) was used to assess the antimalarial activity of the extracts. This strain was kept alive by weekly

re-infection in Swiss mice following the recommendations given by WHO for these type of studies.

- Experimental animals: Male and female mice (*Mus musculus* Swiss) weighing 22-24g originally obtained from the University of Costa Rica were used through the experiments. The animals were maintained in an air-conditioned environment in the animal house of UCIMED, feeding with standard pet food and drinking water *ad libitum*. They were caged in groups of 5-10 and all studies were performed in agreement with international rules for laboratory animal use, under the ACCMAL control in Costa Rica.
- Inoculum: Parasitized erythrocytes were obtained from a previously infected mouse by cardiac puncture using heparin as anticoagulant and then diluted in RPMI 1640 medium, supplemented with 10% fetal calf serum plus antibiotics (RPMI 1640). Animals were inoculated by intraperitoneal injection with a blood suspension (0.2 mL) containing  $10^6$ - $10^7$  parasitized erythrocytes. After 24h infection, mice showing a parasitic presence in blood of ten to 15% of the erythrocytes infected with ring stages, were used in all the *in vitro* studies.
- *In vitro* antimalarial assays: We followed general procedures for *P. berghei* culture and also for testing *in vitro* antimalarial effect (Ager *et al.* 1984, Deharo *et al.* 2000). Briefly, erythrocytes (final cell suspension 1%, v/v, blood/culture medium), from a one day infected mice, suspended in RPMI 1640 medium, were mixed with the intact plant extracts (0.05mL blood and 0.05mL diluted extract per well), the mixture was transferred to plates and then incubated in a candle jar at 37°C for 24h in a low speed shaker (Mini-Shaker 3D Boeco, Germany). For each experiment we used a positive control with chloroquine in standard concentration and a negative control with only culture medium.

To determine the IC<sub>50</sub> (minimal concentration that inhibits 50% of parasite schizogony), we made serial dilutions of the extract according to the dry weight obtained in each case. After the incubation period, we made blood smears from each well, stained with Giemsa dye, and the number of schizonts was determined by counting at least 500 red blood cells under the microscope's 100X objective. The percentage of the parasite schizogony inhibition was calculated by the following formula (Deharo *et al.* 2000):

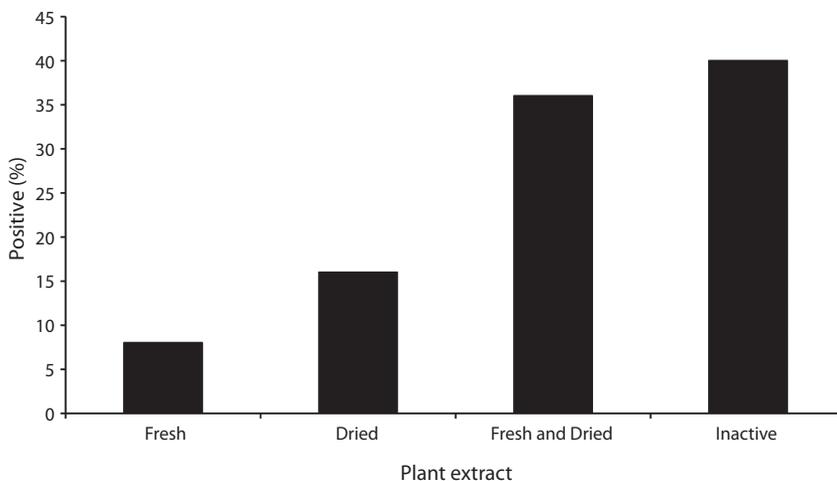
$$\text{Schizogony inhibition (\%)} = \frac{\text{schizonts in control} - \text{schizonts with extract}}{\text{schizonts in control}} \times 100$$

For calculations and statistical analysis the Probit method (Deharo *et al.* 2000, Diaz *et al.* 2004) was used. To classify the activity levels of each extract the Rasoanaivo table (1999) was used. Accordingly the term "very active" means a IC50 less than 5µg/mL; *active* from >5-50µg/mL; "weakly active" from >50-100µg/mL and "inactive" more than 100µg/mL.

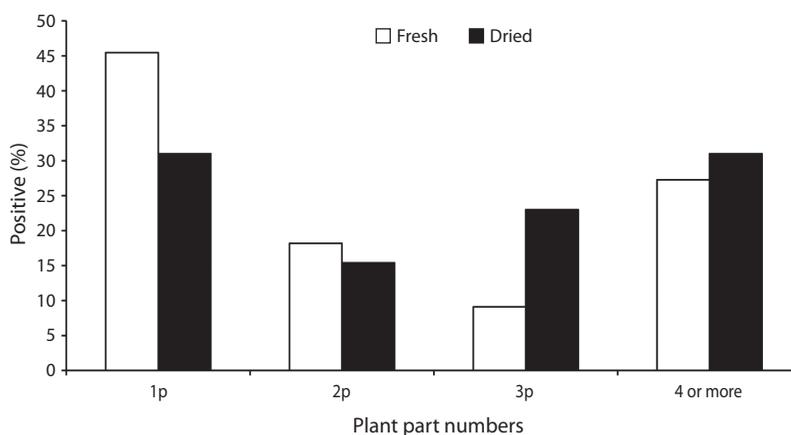
## RESULTS

The crude alcoholic extracts obtained from the different plant structures of the 25 varieties studied (Table 1) showed some antimalarial activity (Fig. 1). The plants with the highest activity consistently showed it in both fresh and dried material. Not all plant structures showed the antimalarial effect. Figure 2 show the percentages of plants with one, two, three, four or more structures with potential effect against *P. berghei*.

There were more plants showing antimalarial activity in only one type of structure, (Figs. 2, 3) but three plants showed it in four or more parts. When comparing extracts of fresh and dried plant with antimalarial activity (Fig. 3), no significant differences were found for structures, except in flowers and young leaves where the dry material showed higher antimalarial activity. Root fresh extracts showed more activity as well.



**Fig. 1.** Percentage of plants with antimalarial activity from the REBAMB according to the type of extract studied.



**Fig. 2.** Distribution of plants according to the number of parts in each of them that showed antimalarial activity: fresh and dried extracts.

IC<sub>50</sub> tests on fresh (Table 2) and dried extracts (Table 3) yielded the results presented in figure 4. Fresh material (Fig. 4A) showed the highest antimalarial activity (“very active”) as compared to the other categories; it was also independent of the part of the plant studied. In contrast, for dry samples (Fig. 4B), the “active” category presented higher values than the others, again regardless of the plant structure studied.

## DISCUSSION

Several authors have pursued the search for natural products with antimalarial effect in plants in the past and in recent years (Rafatro *et al.* 2005, Ogbonna *et al.* 2008, Pillay *et al.* 2008, Kayser *et al.* 2003). The rationale for such studies is based on the resistance of the parasites to conventional treatment as observed in the case of malaria (Fidock *et al.* 2008).

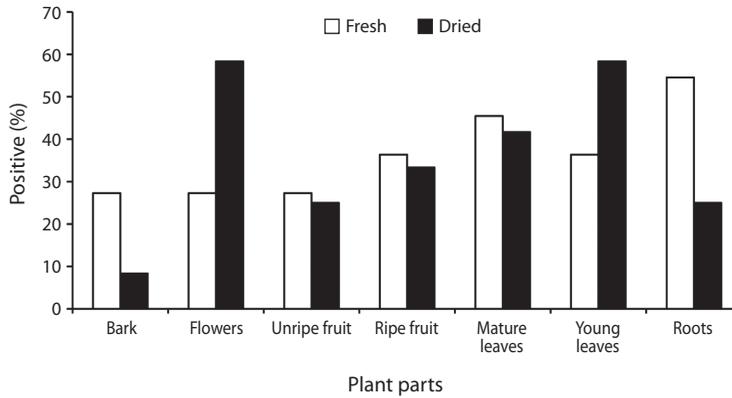


Fig. 3. Relationship between the antimalarial activity found in fresh and dry extracts of positive plants.

TABLE 3  
*In vitro* activity IC<sub>50</sub> (µg/mL) on *P. berghei* of dried extracts

Species	B	F	UF	RF	YL	ML	R
<i>B. frutescens</i>		16.9	5.5	8.8	17.2		
<i>C. chavarrianum</i>	38.7		10.5		7.4	39.8	
<i>C. dioica</i>						19.1	
<i>H. appendiculata</i>		5.3					
<i>N. membranacea</i>		54.3	8.2		1.6	3.8	
<i>N. lobata</i>		4.3			8.9		
<i>P. povedae</i>	20.1			5.4	16.5	34.4	31.8
<i>P. guajava</i>		1		6.2		8	
<i>R. glabra</i>					41		39.1
<i>S. papillosa</i>		19.9				14.1	14.3
<i>S. thecaphora</i>		12.8	9.8		15.5		
<i>S. arboreum</i>				3.5			
<i>W. solanacea</i>					1.6		

B: bark, F: flowers, UF: unripe fruit, RF: ripe fruit, YL: young leaves, ML: mature leaves, R: root.

This resistance began with *P. falciparum* near the '60s and today is extremely common, especially in Africa, Central and South America (Bloland 2001). This resistance has also been documented for *P. vivax* (Baird 2004, Anstey *et al.* 2009) the most common species found outside Africa (Wells *et al.* 2010) and in Costa Rica, were reports account for 95-98% of the cases (Vargas 2001).

Malaria parasites also exhibit resistance to Fansidar, a treatment involving Sulfadoxine

and Pyrimethamine, that act inhibiting the formation of nucleic acids (Bloland 2001, Hastings 2004, Kakkilaya 2008).

Based on the foregoing, we started in Costa Rica the search for antimalarial components in plants, as previously documented (Chinchilla *et al.* 1998, 2001, Castro *et al.* 1996).

In conducting this research, we took into account not only the traditional observations on the treatment of malaria and its symptoms, but also the findings of a previous study

TABLE 2  
*In vitro* activity (IC<sub>50</sub>, µg/mL) on *P. berghei* of plant fresh extracts

Species	B	F	UF	RF	YL	ML	R
<i>A. tridentata</i>						4.9	25.1
<i>B. frutescens</i>	5.9	2.8	2.4	4.6	15.2	2.2	2.6
<i>H. appendiculata</i>							3.9
<i>N. membranacea</i>	71.9	2.2		10.7	0.8		3.2
<i>N. lobata</i>					4.1		
<i>P. povedae</i>						9.7	
<i>P. guajava</i>			5.3			8.2	
<i>S. thecaphora</i>	2		1	3.9			50.8
<i>S. papillosa</i>				0.9			
<i>T. incognitum</i>					3.3	9.1	7
<i>W. solanacea</i>		1.3					

B: bark, F: flowers, UF: unripe fruit, RF: ripe fruit, YL: young leaves, ML: mature leaves, R: root.

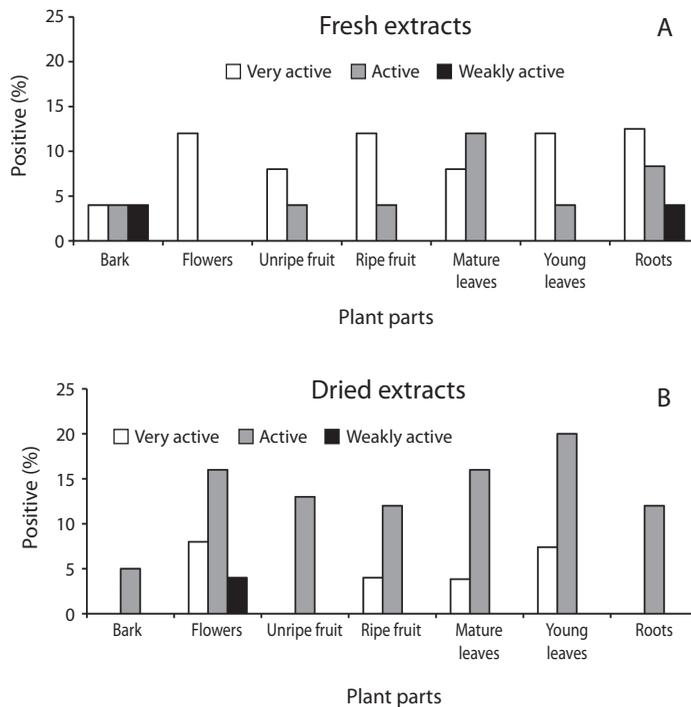


Fig. 4. Relationship between the strength of antimalarial activity and plant parts studied.

(Chinchilla *et al.* 2008), in which, antimalarial activity was found in extracts of certain arthropods, especially those that feed on plants during any stage of its life cycle. The plants found as a feeding source of these insects, guided us in choosing species or similar families as the ones studied in this investigation. This selection system led us to obtain 60% positivity in the 25 plants tested.

Very few papers in other countries mention plants with higher antimalarial activity than the ones found in this work. It could be explained by the fact that these plants are characteristically from Central America (Jenett-Siems *et al.* 1999), and no other researchers have done any work with them.

*H. appendiculata* is found only in Panama, Honduras and Costa Rica (Tropicos 2010) and *T. incognitum* is a recently classified plant that has not been tested for antiparasitic activity. *S. thecaphora*, *N. membranaceae* and *B. frutescens* have a greater distribution in the Americas, but were not found in any other places (Tropicos 2010). In Africa and Asia there has been an intense and enthusiastic search for active compounds against *P. falciparum* especially (Aderounmu 2002, Mariath *et al.* 2009, Titanji *et al.* 2008); the opposite is true in America where many of the studies come from South America (Krettli *et al.* 2001, Rodríguez-Pérez *et al.* 2006, Batista *et al.* 2009, Valadeau *et al.* 2009 among others) and very few from Central America (Franssen 1997, Kohler *et al.* 2002). Antimalarial components have been found in *S. andina*, *S. pauciflora*, *S. tonduziana* and *S. aspera*, but not in *S. thecaphora*. The present study represents an important contribution to this field since the data obtained, together with that already reported, has shown to be inedited.

Most of the studies mentioned, have focused their attention in just a few parts of the plant, but we studied every plant structure, included bark, roots, leaves and fruits in order to optimize the results.

Almost every one of the studies referred before used dried material for extraction. Since the traditional way of using plants for healing is by using fresh material (Blair & Madrigal

2005, Togola *et al.* 2005) we included this modality as part of our research. Shows that the activity in different structures of the plant was very similar independently of the use of the dry or fresh extract. However, when analyzing the potency of IC50, the fresh material showed, except for bark and mature leaves, a dominance of the condition "very active" over "active". The opposite is true for dry extracts; it can be deduced that although the antimalarial activity was detectable in both types of extracts, the potency of this activity appears to decrease by drying the structure. The implication of this observation concludes that if only dry samples were to be studied, some plants with antimalarial activity could be missed.

Since this study was performed in a biological reserve, there is a guarantee for the permanence, for many years, of plants with active components against malaria. In addition, all samples were collected in a fairly restricted area, which reduces the risk of changes due to external factors such as climate, soil type and other (Martin 2001). Factors such as stage of development of the plant and the presence of arthropods, also might affect the data, since the plant needs to synthesize new metabolites in order to defend from them (Coley & Barone 1996). These metabolites could also be active against malaria parasites, but this will be the subject of analysis in other publications.

This work seeks to offer a contribution to Ethnobotany applied to Medical Sciences in Costa Rica, as has been done in many other countries (Martin 2001). Studies in progress are being made for identifying the chemical component(s) responsible for the antimalarial effect of the plant extracts.

#### ACKNOWLEDGMENTS

This study was supported in part by a grant (Proyect Forinves 18) of the Consejo Nacional para Investigaciones Científicas y Tecnológicas (CONICIT), Research Department of the Universidad de Ciencias Médicas (UCIMED) and Centro Regional de Occidente, Universidad de Costa Rica. Special thanks to Laura

Valerio, Jose Bolaños, Edwin Valenciano and Hugo Pérez as well as to some students from UCIMED for their work in the development of all project phases.

## RESUMEN

El tratamiento con las drogas antimaláricas de uso común han inducido resistencia por parte del parásito, lo que obliga a buscar en las plantas de los bosques, componentes naturales con actividad en contra de esta enfermedad. Por lo tanto, decidimos buscar dichos componentes en plantas de una Reserva Forestal de Costa Rica. Extractos tanto frescos como secos de raíz, corteza, hojas, flores y frutos, de 25 plantas de la Reserva Biológica Alberto Manuel Brenes (REBAMB), fueron estudiados *in vitro* en busca de sustancias con actividad antimalárica. Las plantas estudiadas fueron: *Aphelandra aurantiaca*, *A. tridentata* (Acanthaceae); *Xanthosoma undipes* (Araceae); *Iriartea deltoidea* (Arecaceae); *Neurolaena lobata* (Asteraceae); *Senna papillosa*, *Pterocarpus hayessi*, *Lonchocarpus pentaphyllus* (Fabaceae); *Nectandra membranacea*, *Persea povedae*, *Cinamomum chavarrianum* (Lauraceae); *Hampaea appendiculata* (Malvaceae); *Ruarea glabra*, *Guarea glabra* (Meliaceae); *Psidium guajava* (Myrtaceae); *Bocconia frutescens* (Papaveraceae); *Piper friedrichsthali* (Piperaceae); *Clematis dioica* (Ranunculaceae); *Prunus annularis* (Rosaceae); *Siparuna thecaphora* (Siparunaceae); *Solanum arboreum*, *Witheringia solanacea* (Solanaceae); *Ticodendrum incognitum* (Ticodendraceae); *Heliocarpus appendiculatus* (Tiliaceae) y *Myriocarpa longipes* (Urticaceae). Los extractos frescos y secos de las diferentes partes de las plantas fueron estudiadas y se determinó la IC50, el cual osciló entre 1-71.9mg/mL; los extractos frescos mostraron mayor actividad antimalárica. Las plantas que presentaron mayor actividad son muy comunes en Centroamérica y algunos géneros similares, aunque no las mismas especies, han sido encontrados positivos en América del Sur; por esta razón consideramos importante estos resultados como información y materia de discusión en este tema. Además este es el primer estudio sistemático de esta naturaleza realizado en un área boscosa circunscrita y protegida de Costa Rica.

**Palabras clave:** malaria, antimaláricos, *in vitro*, plantas, Costa Rica, *Plasmodium berghei*.

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