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Phytochemical Study on *Ticodendron incognitum* (Ticodendraceae) and Determination of its Antimalarial Activity *in Vitro*

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Abstract

Despite the worldwide efforts to eradicate malaria, it continues to be a global health problem, which is why we are currently searching for active components of natural origin.

Ticodendron incognitum the only species of the Ticodendraceae family was discovered in Costa Rica in 1989 and currently there are no reports on its chemical composition or biological activity. Therefore, the objective of this study was to perform a phytochemical analysis of the crude extract, the root, bark and leaves of the plant, and the determination of the antimalarial activity of the fractions obtained from this phytochemical detection. The qualitative analysis showed the presence of terpenes, coumarins, anthocyanins, flavonoids, phenols, tannins, reducing compounds, triterpenes and sterols. In addition, IC₅₀ values lower than 50 µg/ml, considered as active against the strain of *P. berghei* NK65, were obtained in the ethereal extract of the root and bark and in the hydrolyzed aqueous extract of the root.

This type of studies supports the interest in continuing to identify new components that can be used as a possible treatment for malaria. Based on the results obtained, it brings us to carry out studies in the purification and characterization of the active components, as well as *in vivo* studies that we have already begun, that will allow us to confirm these interesting findings.

Keywords: *Ticodendron Incognitum*; Phytochemical Analysis; Malaria; *Plasmodium Berghei*; *In Vitro* Studies

Introduction

Human malaria is a disease that affects people around the world; even though it is treatable and avoidable, its transmission, morbidity, and mortality are still present in 97 countries. For example, in 2016, there were 216 million cases of malaria in 91 countries, an increase of approximately 5 million new cases over the previous year, 2015. Of those infected, about 450,000 died each year, with the majority being children less than 5-years-old [1]. Malaria-reduction goals proposed for 2020 by World Health Organization (WHO) will not be reached, because global preventive measures have proved difficult, if not impossible, to achieve. In Costa Rica, this disease had been eradicated gradually and by 2014 only six cases were known in the country. However, in 2018, 21 cases have been registered so far, and all of them seem to be imported. This situation is mainly due to the migratory increase of Nicaraguans, due to the political situations in their country [2].

Since 1940, chloroquine has been used as the main antimalarial treatment but, unfortunately, *Plasmodium falciparum*, the most pathogenic of the four species capable of infecting humans, has become resistant to this drug [3]. Therefore, other drugs or combinations of alternative drugs and natural products have been sought for which the human *Plasmodium* species do not yet present resistance. This has led to the discovery and study of several components present in the Chinese plant, *Artemisia annua*, recommended today for the treatment of malaria by the WHO and this has stimulated similar studies in other plants [4,5]. Specifically in Costa Rica, previous studies with plant extracts have generated important contributions in fact some secondary metabolites with antimalarial potential have been identified [6]. Recently, in a study of 25 plants of the Reserva Biológica Alberto

Manuel Brenes (REBAMB), it was demonstrated that the extracts of the leaves of *Ticodendron incognitum* had important activity against malaria [7]. This little-studied plant species is endemic in Central America and was described as a new genus and species for the first time in 1989; therefore, their secondary metabolites, probably responsible for the aforementioned activity, are unknown [8]. Thus, it is important to know such secondary metabolites and here, for the first time, metabolites present in the leaves, roots and bark of *T. incognitum* are described, and their antimalarial activity is evaluated [9].

Materials and Methods

Collection of the plants

The plant was collected in the REBAMB, (number 8 in the herbarium of the UCIMED), which belongs to the University of Costa Rica. REBAMB is located 42 km Northwest of San Ramón, (10° 13' 49" N, 84° 36' 10" W), Alajuela, Costa Rica, with an altitude that varies from 600-1640 m above sea level and with an average temperature of 21 °C, the relative humidity is 98%, and the precipitation of 2461 mm per year [10]. The leaves, root, and bark were placed in new plastic bags and transported to the laboratory according to protocols previously described by Chinchilla *et al.* [11].

Preparation of the extracts

The methods have been previously described in detail by Chinchilla *et al.* [11]. A portion of the bark and leaves of the plant were studied fresh, while a portion of the root was dried at 40 °C for 5 days; 15 g of each of the parts were macerated in 70% ethanol for one week without exposure to light. The hydroalcoholic extracts were filtered under vacuum, using a Whatman 1 filter paper, concentrating them at 40 °C in a rotary evaporator (Buchi R-114) until the ethanol was completely eliminated.

Phytochemical screening

The phytochemical screening of the extract was carried out as established by Sharapin, with some modifications. The procedure was as follows: 40 mL of the crude extract was mixed with 10 mL of distilled water; subsequently, three liquid-liquid extractions with ethyl ether were carried out [12]. The ethereal extract obtained was concentrated to dryness and was called (E). The aqueous extract obtained from the liquid-liquid extraction was separated into two different containers; one of the parties was called AQ₁, conserving it as such, while the other part was hydrolyzed with 10 mL HCl 3 mol/L for 15 min. The hydrolyzed extract was again made a liquid-liquid extraction with 3 x 10 mL of ethyl ether, obtaining a second ether extract (AQ₂) and the residual aqueous extract (AQ₂-hydrolyzed). Each of the extracts were subjected to the corresponding qualitative tests to determine the secondary metabolites present in each part of the extract, as mentioned by Sharapin (2000), to the ethereal phases (E and AQ₂) the chemical tests were applied to them. As result this analysis we determinate the presence of: terpenes (Vainillina), alkaloids (Dragendorff), flavonoids (Shinoda), coumarins (KOH), triterpenes and sterols (Lieberman-Burchard), quinones (Bornträger-Kraus). To the aqueous sample (AQ₁) the presence of: phenols-tannins (FeCl₃), polysaccharides (Lugol), reducing sugars (Fehling), saponins (foam), and alkaloids (Dragendorff) was determined. The hydrolyzed aqueous sample (AQ₂-hydrolyzed) was also determined the presence of anthocyanins by means of an acid-base test. Samples of extracts E, AQ₁ and AQ₂ were prepared to analyze their antimalarial activity. For this, a known amount of the sample was weighed and dissolved in a 10% solution of DMSO, as mentioned in the literature [13]. The extracts that showed activity were the E and AQ₂ of both the root and the dry bark, so some of the metabolites present in this extract is responsible for the antiparasitic activity, which highlights the presence of coumarins, flavonoids, anthocyanins, terpenes, triterpenes and sterols.

Studies of antimalarial activity

For this study, the methodology was described by Chinchilla *et al.* [11]. The animals used were male and female mice (*Mus musculus Swiss*). The *Plasmodium* strain used was NK 65 *P. berghei* from the American Type Culture Collection (ATCC). This strain is maintained in our laboratory by intraperitoneal inoculation (i.p.) weekly in the mice. *Plasmodium berghei* is a species of murine malaria recognized by WHO and the Pan American Health Organization (PAHO) as the model suitable for this type of analysis [14]. All handling of animals was carried out using approved ethical procedures and the corresponding permits (CICUA-07-10) by our two assistants, Laura Valerio Campos and José Bolaños Jiménez, who hold the certificate of Technician in handling of laboratory animals extended by the University of Costa Rica. *P. berghei* trophozoites were cultured at 37 °C in mice red cells, and treated with different concentrations of plant extracts for 24 h, besides the corresponding positive and negative controls. After this incubation period, slides of all the cultures were prepared, fixed and Geimsa stained. The major or minor schizont presence in slides from controls and treated cultures were compared, in order to evaluate any inhibitory effect. The toxicity was determined by the possible red cell lysis caused by each extract. Statistical analyzes of the minimum concentration capable of reducing parasitemia in animals by 50% (IC₅₀) were carried out following the provisions of Deharo *et al.* [15] and by the Probit method [16]. We graded the IC₅₀ as follows: < 10 µg/mL: very active; 10-50 µg/mL: active; 50-100 µg/mL: slightly active; and >100 µg/mL: inactive [17].

Results and Discussion

Ticodendron incognitum, first identified in 1989 in Costa Rica, is the only member of the Ticodendraceae but is found throughout

southern Mexico (Veracruz, Oaxaca, Chiapas) and in southern Central America as far as Panama [8]. It is a little studied species, mainly because it is found in forests that are rarely visited and because of its non-striking appearance [8]. It is why, this species was chosen for these experiments, because to date there are no studies that demonstrate the type of chemical compounds present in each of its parts, and even less research on the possible application and use of its naturally-derived chemicals to human health. This lends special importance to the results obtained in our phytochemical study, where we demonstrate for the first time the effect of some of these chemical extracts on the murine agent, *P. berghei*.

In 2012, Chinchilla *et al.*, reported that the crude extract of the bark, root and tender leaves of *T. incognitum* showed antimalarial activity in the range of 30-120 µg/mL in their *in vitro* tests. According their results, we worked with the leaves, the root, and the bark of the tree for having greater antiparasitic activity. Phytochemical screening was carried out on each of the selected parts to determine the main secondary metabolites present, which could be the cause of the antimalarial activity; compounds we observed included: terpenes, coumarins, anthocyanins, flavonoids, reducing compounds, triterpenes and sterols in their general composition (Table 1).

Metabolite	Part of the plant		
	fresh Leaves	fresh Root	dry Bark
Terpenes	+++	+++	+++
Alkaloids	-	-	-
Flavonoid	-	++	+++
Coumarins	+++	++	+++
Triterpenes and sterols	+++	+++	-
Quinones	-	++	+
Phenols and tannins	+++	-	++
Polysaccharides	+	-	-
Reducing compounds	+++	+++	-
Saponins	+	-	-
Anthocyanins	++	++	++
+++ presence in abundant quantity, ++ presence in moderate amount, + presence in small amount, - absence of metabolite.			

TABLE 1. Phytochemical screening of extracts of leaves, root, and bark of the plant *Ticodendron incognitum*

Similarly, the IC_{50} was calculated for each of the separated extracts according to the corresponding antimalarial activity, showing that the ethereal extract of the root and bark and the AQ_2 of the root were cataloged as assets with IC_{50} of 17.6, 25.9 and 14.9 µg/mL, respectively (Table 2). However, the AQ_2 extract of the bark was classified as not very active, since it presented an IC_{50} of 54.4 µg/mL. The rest of the extracts showed no activity. None of the extracts showed any toxicity red cell, at the active concentrations.

Part of the plant	Extract	Metabolite present in greater quantity	<i>P. berghei</i>
			IC_{50} (µg/mL)
fresh Root	E	Terpenes, triterpenes and sterols	17.6
	AQ_2	Flavonoid, quinones, triterpenes and sterols	14.2
dry Bark	E	Terpenes, flavonoid and coumarins	25.9
	AQ_2	Flavonoid and anthocyanins	54.4
Note: E (ethereal phase); AQ_2 (ethereal phase of hydrolyzed AQ_1).			

TABLE 2. Antimalarial activity (IC_{50}) and secondary metabolites present in the different extracts of the root and bark of the plant *Ticodendron incognitum*

By grouping the results obtained in phytochemical screening and our *in vitro* tests, we determined that one or several of the compounds, terpenes, coumarins, flavonoids, anthocyanins and triterpenes-sterols found in root and bark, can be responsible for the antimalarial activity. Through the analysis of our previous studies and those of others, can confirm that the aforementioned metabolites have been reported in several investigations and in many of the cases they showed antimalarial activity. For example, the studies by Pingaew *et al.* [18], showed that some compounds of the coumarin-chalcone type have a high antimalarial activity with an IC_{50} of 1.6 µg/mL, and that this may be due to an inhibition by this type of compounds to the activity of the *P. falciparum* parasite. Another example of natural antimalarial is 5,7-dimethoxy-8-(3'-hydroxy-3'-methyl-1'-butene-coumarin) isolated from the root of *Toddalaia asiatica*, a compound that presented an IC_{50} of 16.2 µg/mL [19]. Also from the bark of the stem of the plant *Erythrina abyssinica* and the root of *Bidens pilosa* found in Brazil were isolated flavonoids with antimalarial activity [20,21].

Among the triterpenes that have been isolated with antimalarial activity are balsaminoside and karavilagenin extracted from *Momordica balsamica*, which in studies conducted in Brazil, gave inhibition against *P. berghei* [22]. The tricyclic betulinic acid

found in plants such as *Zataria multiflora* and *Zizyphus vulgaris*, as well as its derivatives, also have presented good results in antimalarial activity because some of their compounds are incorporated into the lipid bilayer of erythrocytes, generating modifications in the concentrations of cholesterol, a compound that plays a very important role in parasite vacuolization [23]. Other studies performed by Ngarivhume, van't Klooster, Jong & Van der Westhuizen [24], with medicinal plants used for the treatment of malaria in Zimbabwe, determined that many of these plants are rich in coumarins, flavonoids and triterpenes, among other types of compounds.

Due to the absence of ethnobotanical studies of this plant, it was of interest to report for the first time the phytochemical study for *T. incognitum* species and, in addition to be able to present which metabolites could be responsible for said antimalarial activity.

Conclusion

The phytochemical components found in extracts of bark and root of the plant *T. incognitum*, presented antimalarial activity as shown in some *in vitro* analysis. This finding could provide some scientific and promissory evidence that this plant could prove useful, should further studies warrant, in the management of malaria. It is necessary to perform additional research in our laboratory, to complete the structural elucidation of these active compounds.

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