

# Antimalarial Activity of an Alkaloid Mixture Isolated from the Tender Leaves of the *Nectandra membranacea* (Laureaceae)

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**Abstract:** Despite the breadth of knowledge about malaria, its transmission, preventive measures and available treatments and its difficulty in controlling the vector and the rapid spread of resistance to current drugs, there is a stimulus to develop new research looking for active molecules. The phytochemical studies of the active extract confirmed the presence of alkaloids, flavonoids, coumarins, tannins, reducing compounds, anthocyanins, terpenes, triterpenes and steroids. In the present work, it was possible to isolate a mixture of isoquinolinic alkaloids from the tender leaves of *Nectandra membranacea* (Laureaceae) with antimalarial activity. The mixture composed of (-) - gliazovine and (+) - aplogliazovine showed an IC<sub>50</sub> value of 32.18 µg/mL against the *P. berghei* NK65 strain. It is important to continue with these studies and their chemical components isolated from *N. membranacea* that could be used as an alternative the treatment of malaria.

**Key words:** Malaria, *Nectandra membranacea*, alkaloids, phytochemical screening, Laureaceae.

## 1. Introduction

Malaria is a disease caused by the parasite of the genus *Plasmodium* and transmitted by a mosquito of the genus *Anopheles*. The species *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most cases of infection in humans [1]. Especially, the pathology caused by *P. falciparum* exerts devastating effects on health, affecting large tropical and subtropical populations of the world [2].

Although in recent years, it has been shown statistically that cases of death from malaria have been decreasing, due to controls in prevention and treatment, in 2015 more than 212 million cases were reported, with an estimated of 429,000 deaths attributed to this disease, a situation that worries worldwide [3, 4].

Faced with this situation, WHO has established a strategy to eradicate this disease by 2030, by promoting research and generating the knowledge required to accelerate progress towards a world without malaria [2].

A challenge that currently exists is the search for new antimalarial drugs, because the drugs traditionally used against malaria in endemic regions, such as chloroquine and sulfadoxine-pyrimethamine, have given rise to resistant parasites [5, 6].

For example, regions such as Myanmar, Thailand and Cambodia have forced medical authorities to change the last line of defense, by synthetic derivatives of artemisinin [7].

According to the data obtained between 2012 and 2015 Costa Rica was on the verge of declaring malaria as an eradicated disease; however, in the middle of this year, it was necessary to declare a health alert due to frequent outbreaks of this disease in the country,

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and the resurgence in bordering countries such as Nicaragua, a situation that poses a constant threat to the recovery and resurgence of this disease along with the need to achieve detection and rapid containment of transmission in areas of high risk [8].

Faced with these situations, the importance and the need to search for new antimalarial drugs have allowed the development of different studies at the national level to find plants with promising activity [9-11]. However, these studies have been based on reporting the activity of crude extracts, being of great interest to determine exactly which metabolites are directly responsible for that activity

In one of the studies by Chinchilla et al. [11] it was reported that the extract of the tender leaves of *N. membranacea* presented a significant antimalarial activity against the NK65 strain of *Plasmodium berghei*. Therefore, the present work was carried out to isolate and identify the metabolite(s) responsible for such activity.

## 2. Materials and Methods

**Collection of the plants:** The study was performed in the Alberto Manuel Brenes Biological Reserve (REBAMB), property belonging to the University of Costa Rica, located 42 km northeast of the city of San Ramón in Alajuela, Costa Rica. This reserve is located between 600 and 1,640 m above sea level, with an average temperature of 21 °C, a relative humidity of 98% and a rainfall of 3,461 mm per year.

Within the reserve, the plant was classified by an expert and in accordance with the previous records made in the REBAMB [12, 13]. Then it was geographically located within the reserve using a global positioning system (GPS) and physically marked by a red ribbon.

The plant samples collection has been described by Chinchilla et al. [11]. Briefly, the equivalent of 1 kg of tender leaves was collected, duly packed, labeled, and placed in a cooler for transport to the laboratory, where it was carefully washed to eliminate external

contaminants. The fresh material was finely cut in order to facilitate the extraction processes.

**Extraction, purification and isolation:** The crude extract was obtained from the maceration of the tender leaves during 7 days with occasional agitation; subsequently it was concentrated in a Büchi R-114 rotary evaporator at 40 °C. Purification was carried out by column chromatography (CC) with extraction equipment with solid phase. Then 100% ethyl acetate with increase in polarity was used as a mobile phase until reaching 100% with methanol. Subsequently, by means of thin layer chromatography (TLC), the presence of alkaloids was identified by means of a Merck silica gel 60F<sub>245</sub> absorbent phase (FS), ethyl acetate:methanol (7:3) was used as the mobile phase (FM). All the samples were tested for antiparasitic activity and it was decided to purify the most active fraction. The final purification was performed by the acid-base technique described by Sharapin [14]; where it was possible to isolate a mixture of alkaloids difficult to separate by available chromatography techniques [11]. The corresponding analyses were performed for its identification by gas chromatography, with Shimadzu brand equipment, model GC-17A, with mass spectrometer detector, split mode; Model QP-5000, coupled with a column of type AT-5 (5% Diphenyl polysiloxane), length 30 m, 0.25 mm ID, as helium trawl gas and ionization detector (electronic impact 70 eV) and as databases: Wiley139, NIST107, and SZTERP.

**Phytochemical study:** The method described by Sharapin [14] was done with the following modifications: liquid-liquid extractions were performed with 40 mL of the crude extract and 15 mL of ethyl ether (in triplicate), obtaining an aqueous phase and other ethereal (E1); to the latter, the corresponding qualitative tests were carried out to identify the secondary metabolites present. Half of the volume of this the aqueous phase was acid hydrolysis with 15 mL of HCl 3 mol/L, with subsequent extraction with ethyl ether, obtaining a new ethereal

phase (E2) and a hydrolyzed aqueous phase. Ethereal phases were applied to chemical tests for the determination of: terpenes (vanillin), alkaloids (Dragendorff), flavonoids (Shinoda), coumarins (KOH), triterpenes and sterols (Lieberman-Burchard), quinones (Bornträger-Kraus). The presence of phenols-tannins ( $\text{FeCl}_3$ ), polysaccharides (Lugol), reducing sugars (Fehling), saponins (foam), and alkaloids (Dragendorff) was determined as the aqueous samples. The presence of anthocyanins was also determined to hydrolyze aqueous sample by means of an acid-base test, as mentioned by the method [14]. To each of the samples obtained after the liquid-liquid extraction, the corresponding antiparasitic tests were carried out to determine which extract exhibited said activity.

**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 parasite strain used was the NK65 of *Plasmodium berghei* from the American Type Culture Collection (ATCC). This strain is maintained in the laboratory in mice by weekly intraperitoneal (i.p.) inoculation. *P. berghei* is a species of murine malaria recognized by WHO and PAHO as the model suitable for this type of analysis [15].

Statistical analyses of the concentration capable of reducing parasitemia in animals by 50% ( $\text{IC}_{50}$ ) were carried out following the provisions of Deharo et al. [16] and by the Probit method [17]. The  $\text{IC}_{50}$  less than 10  $\mu\text{g/mL}$  is considered very active, between 10 and 50  $\mu\text{g/mL}$  active, 50 to 100  $\mu\text{g/mL}$  little active and greater than 100  $\mu\text{g/mL}$  without activity [18].

### 3. Results

The phytochemical screening of the tender leaves of *N. membranacea* shown in Table 1 revealed the presence of alkaloids, flavonoids, coumarins, tannins, reducing compounds, anthocyanins, terpenes, triterpenes and steroids in their general composition.

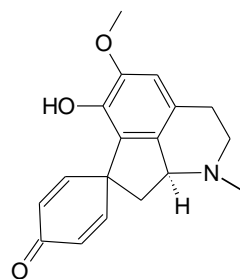
According to the results reported by Chinchilla et al. [19] in relation to the antiplasmodic activity of the

crude extract of the tender leaves *N. membranacea*, fractionation was realized by column chromatography guided by TLC; a total of 58 fractions were obtained. The fraction more active indicated the presence of alkaloids when sprayed with Dragendorff reagent; of them it was possible to isolate two isoquinolinic alkaloids difficult to separate by CC (Fig. 1), which were identified by GC/MS (Gas chromatography-mass spectrometry).

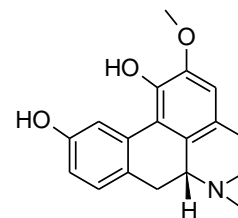
Based on the fractionation pattern of the mass spectra (Figs. 2 and 3), the comparison with the data from the literature and the data obtained by the NIST107 and NIST21 database, two alkaloids of molecular formula  $\text{C}_{18}\text{H}_{19}\text{NO}_3$   $m/z = 297.00$  were found, one such as (-) - gliazovine and a second alkaloid such as (+) - aploglaziovine.

**Table 1** Phytochemical screening of the tender leaves of *N. membranacea*.

Metabolite	Ethereal extract (E1)	Aqueous extract	Hydrolyzed aqueous extract (E2)
Alkaloids	-	+	+
Flavonoids	+	+	+
Coumarins	-	+	+
Triterpenes and sterols	+		+
Quinones	-	-	-
Tannins	-	+	
Reducers compounds		+	
Anthocyanins			+
Terpenes	+		
Saponins	-	-	
+ Presence		-Absence	



compound 1



compound 2

**Fig. 1** Chemical structure of (-) - gliazovine (compound 1) and of (+) - aploglaziovine (compound 2) isolated from the tender leaves of *N. membranacea*.

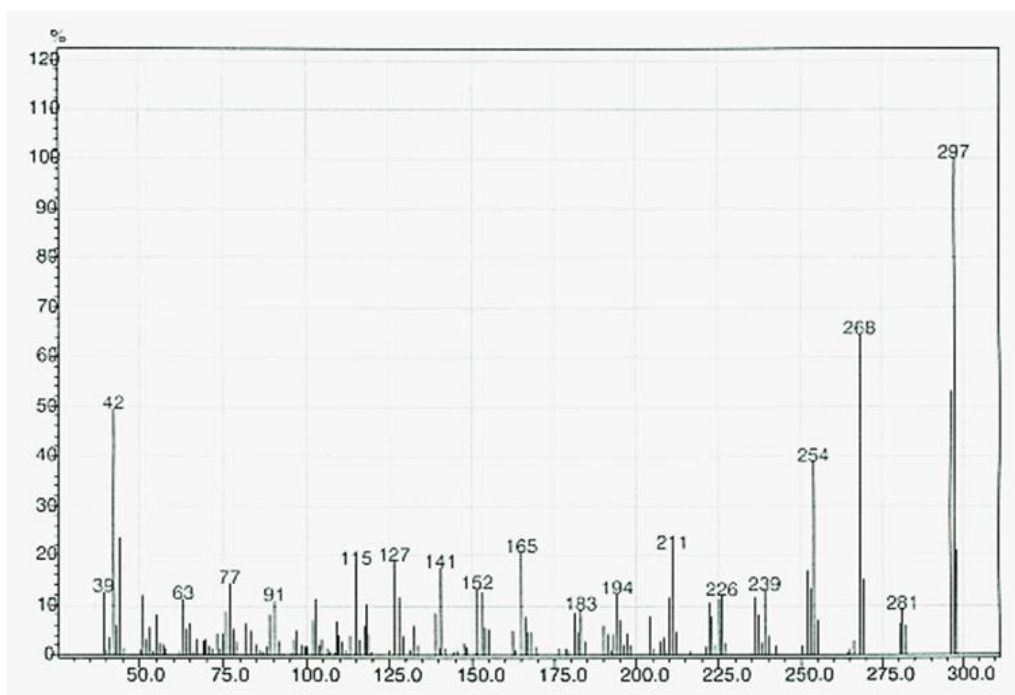


Fig. 2 Mass spectrum of compound 1 ( $C_{18}H_{19}NO_3$  (-)-gliazovine).

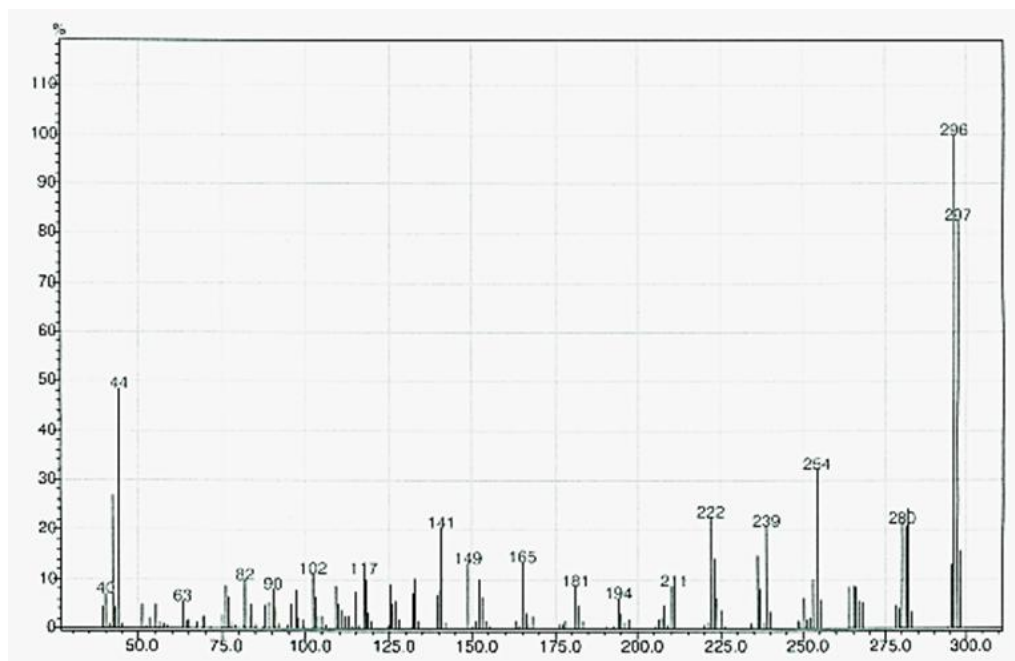


Fig. 3 Mass spectrum of compound 2 ( $C_{18}H_{19}NO_3$  (+)-aploglazovin).

The purified alkaloid mixture showed an *in vitro* antimalarial activity with an  $IC_{50}$  value of 32.18  $\mu\text{g/mL}$  against the strain of *P. berghei* NK65.

#### 4. Discussion

Although mortality rates from malaria have

decreased by 47% globally since 2000 and by 54% in the African region, malaria remains a disease considered by WHO as a global health problem [2, 20]. Data obtained from WHO, indicate that global strategies for malaria control have been effective because in 2016, malaria was considered endemic in

91 countries and territories, compared to 108 countries in 2000. However, prevalence remains high in various areas of Africa, Asia and South America; mainly in children, where a child dies every minute as a result of malaria [21].

Besides that it is very well known the resistance that has been registered for various strains and species of *Plasmodium* to chloroquine and artemisinin, it is why, one of the strategies to face this challenge is the search for plants that contain active antimalarial metabolites that contribute to the development of new treatments for the control of the disease [7, 22].

An etbotanical study by Asnake et al. [23] revealed that the families of the Lamiaceae and Asteraceae provide the highest number of species used as antimalarial medicinal plants, followed by the families Rutaceae, Cucurbitaceae, Euphorbiaceae and Fabaceae in the southern region of Ethiopia.

Another study conducted by Benelli et al. [20], indicates that the phytochemical analyses of twelve plant species, commonly used by local populations in Madagascar to treat malaria, showed the presence of complex alkaloids, which showed efficacy both *in vitro* and *in vivo* against *Plasmodium* strains resistant to chloroquine. The most promising compounds that showed significant effects belonged to the families of the Loganiaceae, Menispermaceae and Rutaceae. However, *Ocotea racemosa* family of Lauraceae was the second most active plant with an  $IC_{50}$  0.9  $\mu\text{g/mL}$ , a family characterized by its high alkaloid content.

According to Grecco et al. [24], *Nectandra* is one of the largest genera in the Laureaceae family and comprises about 120 species that only occur in the tropical and subtropical regions of America, Africa, Asia, and Oceania. Plants of this genus have been used in the treatment of several clinical disorders in humans, including pain, inflammation, fever, hypertension, parasitic infections by *Leishmania* sp., *Plasmodium* sp. and *Trypanosoma cruzi*, among others [24]. This has caused that scientific studies on the genus *Nectandra*, have increased in recent years

due to the different secondary metabolites reported in the different species with biological activity. For example; different several secondary metabolites have been identified in different *Nectandra* species, among which sesquiterpenes of *N. cissiflora* and *N. amazonum* stand out [25, 26], alkaloids in *N. megapotomica*, *N. membranacea*, *N. salicifolia*, and *N. cuspidata* [27-30], lignans and phenylpropanoids in *N. megapotomica* [31, 32] and lactones in *N. gardneri* [33].

Preliminary studies described by Chinchilla et al. [19] can make a valuable contribution about the potentially useful antimalarial compound, an example is *Nectandra membranacea*, which in preliminary studies showed high antimalarial potential. This led to the need to identify which of the various metabolites obtained in the phytochemical study was responsible for the antiplasmodic activity [19].

#### 4. Conclusions

This study shows that the alkaloids (-) - gliazovine and (+) - aplogliazovine are the metabolites responsible for the antimalarial activity of *N. membranacea*, whose mixture has an  $IC_{50}$  32.18  $\mu\text{g/mL}$ , which according to Rasoanaivo et al. [18], can be included as an active compound against *Plasmodium*.

No other studies on these alkaloids have been recorded in relation to antiparasitic effects.

Therefore this study is a new contribution to the treatment of *Plasmodium*, with natural products, a very important issue at this time, given all the work known worldwide, on antiparasitic components in plants.

This type of research allows generating data with scientific basis for the development, innovation and production of natural medicines based on standardized extracts of plant species to be applied in the field of public health.

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