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Research Article

Chemical Composition and *In Vitro* Antiplasmodial Activity of the Total Alkaloids of the Bulbs of Two Amaryllidaceae Species from Northern Peru

Marilú Roxana Soto-Vásquez[1,*], Madeleine Vanessa Horna -Pinedo[1], Luciana R. Tallini[2], Jaume Bastida[3]

ABSTRACT

Introduction: The amaryllidaceae family is characterized by presenting alkaloids with powerful pharmacological activities, including antiprotozoal activity. The aim of the present work was to determine the chemical composition and evaluate the in vitro antiplasmodial activity of the total alkaloids of the bulbs of two amaryllidaceae species from northern Perú. **Methods:** The total alkaloids were extracted from the bulbs using an acid-base extraction. The chemical composition of the total alkaloids was determined by GC-MS, using galantamine as a reference standard. It was investigated the in vitro antiplasmodial activity against *Plasmodium falciparum* FCR-3 strain (chloroquine-resistant). **Results:** 8 alkaloids were identified in the bulbs of *Clinanthus incarnatus*: lycorine, galanthamine, galanthine, vittatine/crinine, hippamine, 3-*O*-acetylpowelline, 11,12-dehydroanhydrolycorine, 1-*O*-acetyllycorine with values of 19.73; 14.99; 10.36; 10.22; 10.16; 10.14; 10.04; 9.85 µg GAL/100 mg of total alkaloid (TA) respectively and 6 alkaloids in the bulbs of *Clinanthus ruber*: lycorine, anhydrolycorine, 11,12-dehydroanhydrolycorine, 2,4-didehydro-2-dehydroxylycorine, 8-0-dimethylmaritidine, hippamine, with values of 70.2; 18; 4.15; 3.45; 6.8 and 0.1 µg GAL/100 mg TA respectively. The total alkaloids of the species of *C. incarnatus and C. ruber* at concentrations of 1.0; 2.5; 5.0; 10.0; 25.0 and 50.0 µg/ml presented inhibition percentages of 23.5 ± 0.46% to 94 ± 0.56% against *P. falciparum* with ($p < 0.05$). They also presented IC_{50} 0.375 µg/ml (*C. incarnatus*) and IC_{50} 0.241 µg / ml (*C. ruber*). **Conclusion:** The main component of total alkaloids of the bulbs of two species was lycorine, in adittion, these species showed in vitro antiplasmoidal activity against *Plasmodium falciparum* FCR-3 strain at the doses tested.

Key words: *Clinanthus incarnatus, Clinanthus ruber, Plasmodium falciparum.*

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INTRODUCTION

Malaria is an infectious and life-threatening disease caused by *Plasmodium* parasites such as *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax and Plasmodium malariae*; Among these protozoa, *P. falciparum* is believed to be responsible for most serious diseases and most fatal cases[1]. In 2018, the World Health Organization (WHO) declared 228 million cases of malaria worldwide; mostly in the African Region, followed by the Eastern Mediterranean, Western Pacific and Southeast Asia; including 405,000 deaths[2]. To face this situation, WHO has recommended the use of therapies based on combinations of artemisinin and derivatives with other drugs; however, in some countries *P. falciparum* is already resistant to artemisinin combination therapies [3].

The Global Technical Strategy for Malaria 2016–2030 defined the goal for 2030 to decrease the 90% of the incidence rate of malaria as well as related death rate[4]. To contribute with this purpose, we need to find new sources of medicaments against this illness. In this sense, plants are the main source of medicinal agents, even a large part of the world's population use herbs and it is not surprising to find a well-established system of traditional medicine in many countries[5]. The recognition and validation of traditional medicine is important and could lead to the discovery of new plant-derived drugs, such as quinine isolated from *Cinchona* species and artemisinin isolated from *Artemisia annua* L[6]. In addition, many compounds from various medicinal plants were isolated and showed *in vitro* and *in vivo* antiplasmodial activity against[7].

The species *Clinanthus incarnatus* and *Clinanthus ruber* belong to the Amaryllidaceae family. This monocotyledonous botanical family is widely distributed throughout the world with approximately 70 genera and 1600 species. Besides there are 28 genera in South America and 24 genera in Peru, finding in this territory 138 species, among which are 15 to 20 species belonging to genus *Clinanthus*[8].

Amaryllidaceae family plants contain, especially in the bulbs, a variety of unique alkaloids not present in other families. These isoquinoline alkaloids have powerful medicinal properties, including antitumor, antiviral, cytotoxic, acetylcholinesterase inhibitory, immunostimulating, anti-inflammatory, analgesic, and for the treatment of Alzheimer's disease[9]. Some of these alkaloids are of particular interest due to their potential antiprotozoal activity such as lycorine, agustinine and crinamine from *Crinum amabile* bulb, in addition, Haemanthamine and 6-hydroxyhaemanthamine exhibited antimalarial activity against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*[10].

There are no previous reports on the species under study, so this research constitutes the first report in this regard. In this way, the objective of the research

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was to determine the chemical composition and evaluate the in vitro antiplasmodial activity of the total alkaloids of the bulbs of two amaryllidaceae species from northern Peru.

MATERIALS AND METHODS

Collection of samples

The bulbs of *Clinanthus incarnatus* (Kunth) Meerow and *Clinanthus ruber* (Herb.) Meerow & A. Cano were collected from the districts of Otuzco (2641masl) and Pataz (3118 masl) in La Libertad Region. The botanical identification was carried out by Dr. Alan Meerow from Agricultural Research Service, United State Department of Agriculture, Miami, FL (USA), and deposited in the Herbarium Truxillense of the National University of Trujillo (HUT).

Extraction of alkaloids

The bulbs were washed, disinfected and cut into thin slices. Then they were dried in a forced convection oven at 40 ºC for 72 hours. Once the plant material was completely dried, it was ground in a rotary blade mill. The dried powdered material was macerated with methanol for 72 hours at room temperature, applying ultrasonic baths at intervals of 1 to 2 hours. Subsequently, the methanolic extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator at a temperature of 40 °C. The crude extract obtained was subjected to acidification with H_2SO_4 (2% v/v) and was cleaned with ethyl ether, separating the organic phase composed of neutral materials such as chlorophylls, waxes and mucilages from the aqueous phase, rich in alkaloids. The acidic aqueous phase was subjected to basification with NH_4OH (10% v/v) until reaching a pH of 10, then the alkaloids were extracted through the repeated use of chloroform, so that the alkaloids were retained in the organic phase. Then the solvent was evaporated under reduced pressure in the rotary evaporator at a temperature of 45 °C, obtaining the extract of total alkaloids (TA)[11-13].

GC-MS conditions

Alkaloids were identified by using a GC-MS apparatus (Agilent Technologies 6890 N coupled with MSD5975 inert XL) operating in the electron ionization (EI) mode at 70 eV. A Sapiens-X5 MS column (30 m x 0.25 mm i.d., film thickness 0.25 µm) was used. The temperature gradient was as follows: 12 min at 100 °C, 100-180 °C at 15 °C/min, 180-300 °C at 5 °C/min and 10 min hold at 300 °C. The injector and detector temperatures were 250 and 280 °C, respectively, and the flow-rate of carrier gas (He) was 1 ml/min. Two mg of each total alkaloids was dissolved in 1 ml of MeOH: $CHCl_3$ (1:1, v/v) and 1 µl was injected using the split-less mode. Codeine (50 µg/ml) was used as an internal standard.

Alkaloid quantification

To quantify the single constituents, a calibration curve of galanthamine (10, 20, 40, 60, 80 and 100 µg/ml) was used. The same amount of codeine (50 µg/ml) was added to each sample as an internal standard. The peak areas were manually obtained considering selected ions for each compound (base peak of their MS, i.e., m/z at 286 for galanthamine and 299 for codeine). The ratio between values obtained for galanthamine and codeine in each solution was plotted against the corresponding concentration of galanthamine to obtain the calibration curve and its equation (y = 0.0224x-0.2037; R2 = 0.9977). All data was standardized to the internal standard area (codeine) and the equation obtained for the calibration curve of galanthamine (GAL) was used to calculate the amount of each alkaloid. Results are expressed as µg GAL/ 100 mg TA (total alkaloid).

In vitro antiplasmodial activity

The evaluation of the antiplasmodial activity of the total alkaloids was carried out in vitro with strain FCR3 (chloroquine resistant) of *Plasmodium falciparum*, which were cultured in RPMI 1640 medium supplemented with 10% human serum and a hematocrit of 4% that was obtained adding 200 µl of total red blood cells in 4.5 ml of RPMI 1640 and 0.5 ml of serum or plasma (Blood group 0, Rh $^+$) and incubated at 37ºC in a 5% O_2 6% gas mixture atmosphere of CO_2 and balanced N_2, as described by Trager W, et al, with some modifications. The tests for the antiplasmodial activity of the total alkaloids (1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 µg /ml dissolved in DMSO), were carried out in 96-well plates of flat bottom, for each alkaloidal extract in triplicate. Chloroquine diphosphate (10 to 1000 nM) was used as a control of the test. The cultures were synchronized with a parasitaemia and a hematocrit of 1 and 2% respectively; These were dispensed in a volume of 100 µl in 96-well plates in duplicate, 100 µl of the total alkaloids were added, and finally they were incubated at 37 ° C for 48 hours. After this incubation time, the upper phase of the culture was completely eliminated, to make a smear of the sediment from each well, then fixing with methanol and staining with Giemsa. These plates were observed under the microscope with a 100x immersion lens, counting uninfected red blood cells (GRL) and infected red blood cells (GRI), to obtain the percentage (%) of Inhibition calculated by the formula[14-16]:

$$\% \text{ inhibition} = \frac{(GRL - GRI)}{GRL} X\ 100$$

The IC_{50} value was calculated by an activity curve: Percentage of inhibition vs. logarithm of drug concentration, through linear interpolation calculation:

$$Log\ (IC50) = Log(X1) + 50 - Y1/Y2 - Y1[Log(X2) - Log(X1)]$$

X1 = Concentration of the drug that gives an inhibition of Y1 parasitemia> 50%;

X2 = Concentration of the drug giving an inhibition of Y2 parasitemia <50%;

Y1 = Percentage of inhibition of X1

Y2 = Percentage of inhibition of X2

Statistic analysis

The results were processed using the statistical program SPSS v. 23 and, expressed as the arithmetic median ± standard deviation. The relationship between the groups was determined using the one-way ANOVA test, in which p0.05 were considered statistically significant.

RESULTS

Alkaloids Identified in *C. incarnatus* and *C. ruber* by GC–MS

The identified alkaloids and their structures are represented in Table 1 and Figure1. The alkaloids present in the analyzed samples were identified by comparing their GC-MS spectra and Kovats retention index (RI) values with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectrometric methods (NMR, UV, CD, IR, MS) in the Natural Products Laboratory of Barcelona University, the NIST 05 Database, or literature data. The MS spectra were deconvoluted by AMDIS 2.64 software (NIST).

As can be seen in Table1 and Figure 1, 8 alkaloids were identified in the bulbs of *Clinanthus incarnatus* using GC-MS: lycorine (7), Galanthamine (11), galanthine(5), vittatine/crinine(8), hippamine(5), 3-*O*-acetylpowelline (9), 11,12-dehydroanhydrolycorine(3), 1-*O*-acetyllycorine(6) with values of 19.73; 14.99; 10.36; 10.22; 10.16; 10.14; 10.04; 9.85 µg GAL/100 mg of TA respectively.

Approximately 87.5% of the identified alkaloids were of the lycorine type, 25% of the crinine/haemanthamine-type and 12.5% of the Galanthamine-type. Meanwhile in *Clinanthus ruber* bulbs, 6 alkaloids were

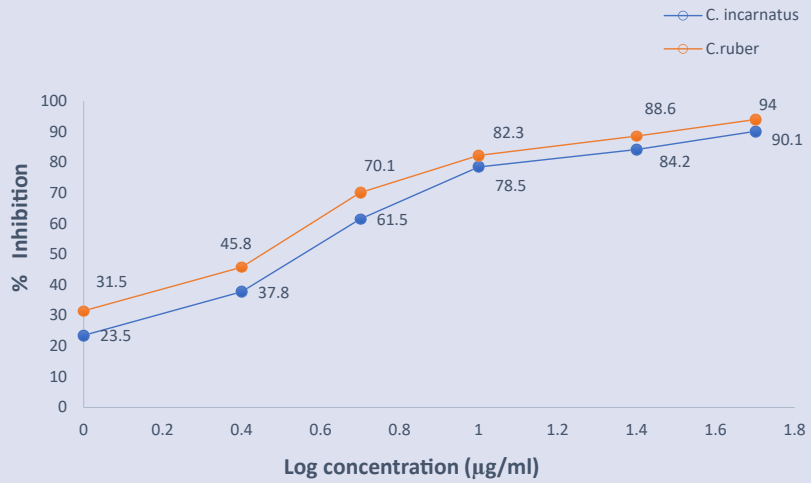


Figure 1: *In vitro* antiplasmodial activity of the total alkaloids of the bulbs of *Clinanthus incarnatus* and *Clinanthus ruber*.

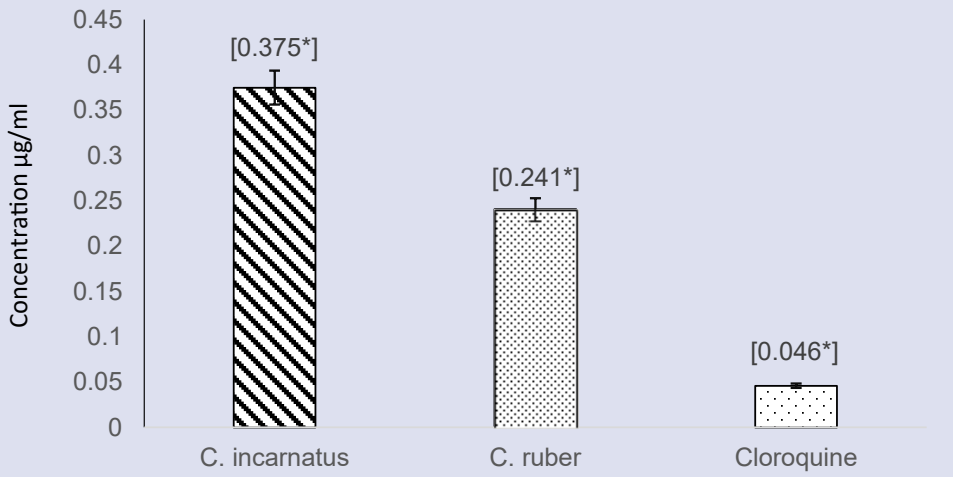
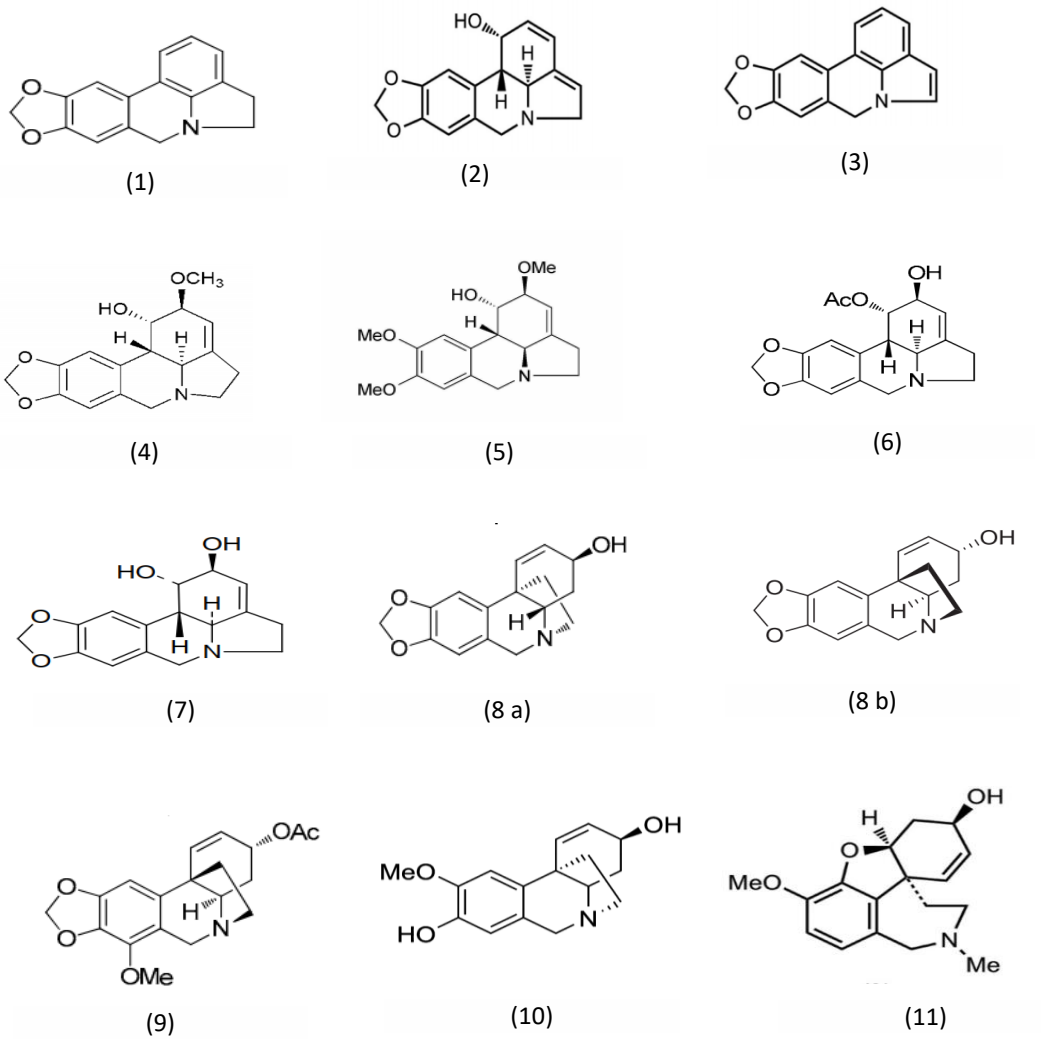


Figure 2: CI_{50} of the total alkaloids (TA) of the bulbs of *Clinanthus incarnatus* and *Clinanthus ruber* and cloroquine.

Table 1: Alkaloids identified in *Clinanthus incarnatus* and *Clinanthus ruber* by GC-MS. Values in µg GAL/100 mg TA.

Alkaloids	[M+]	Rt (min)	RI	C. incarnatus	C. ruber
Lycorine-type					
Anhidrolycorine (1)	251	23.962	2501.6	-	18.0
2,4-didehydro-2-dehydroxylycorine (2)	269	24.451	2534.4	-	4.15
11,12- dehydroanhydrolycorine (3)	249	25.508	2653.2	10.04	5.81
Hippamine (4)	301	26.514	2705.8	10.16	<0.1
Galanthine (5)	317	26.884	2730	10.36	-
1-O-Acetyllycorine (6)	329	27.142	2747.0	9.85	-
Lycorine (7)	287	27.693	2783.1	19.73	70.2
Crinine/haemanthamine-type					
Vittatine /crinine (8)	271	23.660	2518.7	10.22	-
3-O-acetylpowelline (9)	343	25.370	2630.8	10.14	
8-0-demethylmaritidine (10)	273	28.327	2794.2	-	3.45
Galanthamine-type					
Galanthamine (11)	287	22,355	2433,2	14.99	-
Not identified					
NI, m/z 201 ; [M=273] * (12)	273	23.904	2497.8	-	<0.1
NI, m/z 125 (homolycorine-type)* (13)	125	29.419	2867.4	-	6.80

* proposed structure-type according to the fragmentation pattern; Rt: retention time; RI:Kovats Retention Index; NI: not identified.



Alkaloids identified in *C. incarnatus* and *C. ruber* by GC-MS. Anhidrolycorine (1), 2,4-didehydro-2-dehydroxylycorine (2), 11,12- dehydroanhydrolycorine (3), hippamine (4), galanthine (5), 1-*O*-acetyllycorine (6), lycorine (**7**), vittatine(8a)/crinine(8b), 3-*O*-acetylpowelline (9), 8-*O*-demethylmaritidine (10), galanthamine (11).

identified: lycorine(7), anhydrolycorine (1), 11,12-dehydroanhydrolycorine(3), 2,4-didehydro-2-dehydroxylycorine(2), 8-0-dimethylmaritidine(10), hippamine (4), with values of 70.2; 18; 5.81; 4.15; 3.45 and <0.1 µg GAL /100 mg TA respectively; somehow, 2 alkaloids were not identified (m/z 201 [M+ = 273](12), m/z 125 (homolycorine-type) (13), with values of <0.1, 6.80 µg GAL /100 mg TA respectively. From 100% of alkaloids identified, 83% correspond to the lycorine type and 17% to the crinine/haemanthamine type.

Shows that there is a directly proportional relationship between the concentration of total alkaloids and the percentage of inhibition of the *P. falciparum* with values of 23.5 ± 0.46% at 90.1 ± 0.1% (*C. incarnatus*) and 31.5 ± 0.1% at 94 ± 0.56% (*C.ruber*). Besides, in figure 2, the values of IC_{50} 0.375 µg/ml (*C. incarnatus*) and IC_{50} 0.241 µg/ml (*C. ruber*) are shown, indicating antiplasmodial activity. ANOVA and Tukey test were applied, determining that there is a statistically significant difference between the percentages of inhibition and IC_{50} values between both species and chloroquine ($p < 0.05$)

The data presented correspond to the average of three replications ± standard deviation. The asterisks represent a significant difference in relation to the control (chloroquine) according to the ANOVA test ($p < 0.05$).

DISCUSSION

The amaryllidaceae family is distinguished for its exclusive alkaloids isolated from all its genera[17]. Generally are isoquinoline type alkaloids that have not been identified in any other plant family and are classified into nine different types based on the heterocyclic system: norbelladine, lycorine, homolycorine, crinine, hemantamine, narcyclisine, tazetine, montanine and galantamine[18,19].

In this sense, the alkaloids found in both species in this research belong to lycorine type as well as crinine/haemanthamine-type, founding that lycorine is the majority alkaloid in both species with values of 19.73 µg GAL / 100 mg TA (0.01973%) and 70.2 µg GAL /100 mg TA (0.0702%) respectively; what matches other investigations in amaryllidaceae species where concentrations ranged from 0.006% to 0.162% for *Galanthus elwesii*[20], 0.10-0.53% for *Sternbergia sicula*[21], 0.19-0.40% for *Sternbergia lutea*[21] and 0.05-0.14% for *Pancratium maritimum*[21], 0.009% to 0.012% for *Galanthus trojanus*)[22], and 0.004% for *Galanthus cilicicus*[22]. It should be noted that the variability in the concentration depends on the environmental conditions and stress factors to which the plant is exposed[20-22]. Besides lycorine has a variety of biological activities (antineoplastic, immunostimulant,

bacteriostatic, anticholinesterase, analgesic, anti-inflammatory, antiviral, antiprotozoal and antimalarial)[23].

The total alkaloids of the species of *C. incarnatus* and *C. ruber* at concentrations of 1µg/ml, 2.5µg/ml, 5µg/ml, 10µg/ml, 25µg/ml and 50 ug/ml showed inhibition percentages from 23.5 ± 0.46% at 90.1 ± 0.1% (*C. incarnatus*) and 31.5 ± 0.1% at 94 ± 0.56% (*C.ruber*) against *P. falciparum*, obtaining the highest percentages at a concentration of 50 µg/ml with values of 90.1 ± 0.1% and 94 ± 0.56% respectively. Besides these alkaloids presented values of IC_{50} 0.375 µg/ml (*C. incarnatus*) and IC_{50} 0.241 µg / ml (*C. ruber*), and when these results are compared with the criteria of the Research Initiative on Traditional Antimalarial Methods - RITAM, we found that they present a good level of activity[24]. In this context, some studies show that the alkaloids of the bulbs of the amaryllidaceae family present antiplasmodial activity in vitro against *P. falciparum* such as lycorine (IC_{50} 1.026 µg/ml), crinine (IC_{50} 2.110 µg /ml), haemantamine (IC_{50} 0.703 µg/ml), 6-hydroxyhaemantamine (IC_{50} 0.348 µg /ml), 3-epihydroxybulbispermine (IC_{50} 1.139 µg /ml), galantamine (IC_{50} 4.38 µg /ml), tazzetine (IC_{50} 5.420 µg/ml), ismine (IC_{50} > 10 µg/ml), 1-O-acetylcaranine (3.21 µg/ml), 3-O-acetylhamaine (1.14 µg/ml), bufanamine (IC_{50} 25.9 µg/ml)[25,26]. In addition, other amaylliadaceae alkaloids such as haemantamine, haemantidine, lycorine, 3-epihydroxybulbispermine, galantine and pancracine, also showed antimalarial activity against the chloroquine-resistant strain K1 of *P. falciparum*, with values of IC_{50} below 1 µg/ml[27]. Numerous investigations show that lycorine has many properties such as anti-inflammatory, antibacterial, antitumor, antiviral and antimalarial, even it was discovered that lycorine is the most powerful alkaloid against *Plasmodium falciparum*.[27,28] Analysis of the chemical structure and antimalarial activity of lycorine shows that the best antimalarial effect is achieved with derivatives of lycorine that have free hydroxyl groups at C-1 and C-2, or esterified as acetates or isobutyrates. Furthermore, some studies afirmn that C-2-C-3 double bond also plays an important role in the antiplasmodial effect of lycorine derivatives[28,29]. Besides, 1,2-O-diacetyllycorine, other lycorine type alkaloid showed a IC_{50} of 0.097 ug/ml against the F-32 strain[30]. In this way, amaryllidaceae alkaloids, in special, lycorine type alkaloids are an interesting and viable option for drug discovery in antimalarial field, constituing this work in the first report of in vitro antiplasmodial activity for these species, thus contributing to the worldwide need to find new natural sources with antiplasmoidal potential.

CONCLUSION

The main component of total alkaloids of the Bulbs of two Amaryllidaceae species from Northern Peru was lycorine, in adittion, these species showed antiplasmoidal activity in vitro against *Plasmodium falciparum* (strain FCR3 resistant to Chloroquine) at the doses tested.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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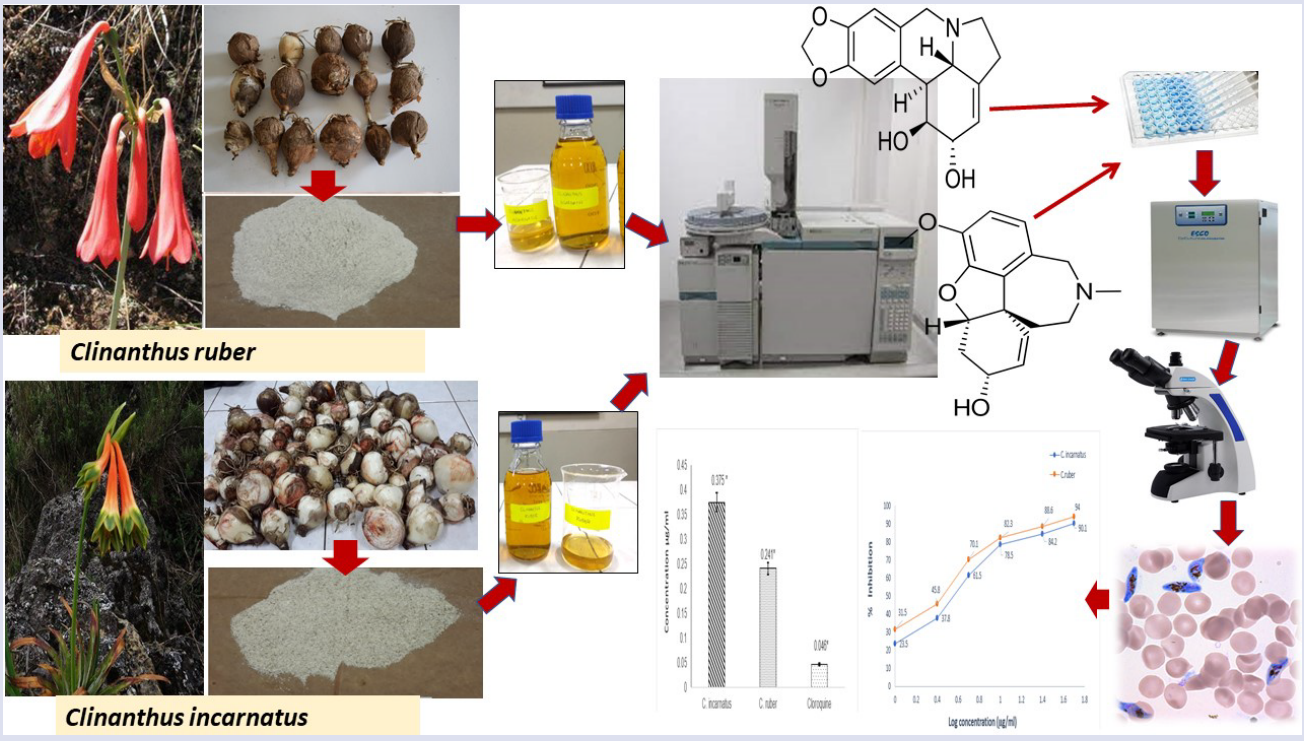
GRAPHICAL ABSTRACT



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