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

Marilú Roxana Soto-Vásquez1,*, Madeleine Vanessa Horna -Pinedo2, Luciana R. Tallini2, Jaume Bastida3

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 antiprotozoal activity of the total alkaloids of the bulbs of two amaryllidaceae species from northern Peru. 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 antiprotozoal 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-dehydroxyhydrocorynine, 1-O-acetylcorynine 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-dehydroxyhydrocorynine, 1-O-acetylcorynine, 2,4-didehydro-2-dehydroxycorynine, 8,0-dimethyltridine, 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 IC50 0.375 µg/ml (C. incarnatus) and IC50 0.241 µg / ml (C. ruber). Conclusion: The main component of total alkaloids of the bulbs of two species was lycorine, in addition, these species showed in vitro antiprotozoal activity against Plasmodium falciparum FCR-3 strain at the doses tested.

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

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 cases1. 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 deaths2. 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 therapies3.

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 rate4. 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 countries5. 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. In addition, many compounds from various medicinal plants were isolated and showed in vitro and in vivo antiprotozoal activity against7.

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 Clinanthus8. 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 disease9. Some of these alkaloids are of particular interest due to their potential antiprotozoal activity such as lycorine, agastinine and crinamine from Crinum amabile bulb, in addition, Haemanthamine and 6-hydroxyhaemanthamine exhibited antimalarial activity against chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falciparum10.

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...
was to determine the chemical composition and evaluate the in vitro antimalarial 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 (2641 m asl) and Pataz (3118 m asl) 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 °C 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 H2SO4 (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 NH4OH (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 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 H2SO4 (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 NH4OH (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).

**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: CHCl3 (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 to obtain the calibration curve and its equation (y = 0.022x-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 antimalarial activity**

The evaluation of the antimalarial 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 37°C in a 5% O2, 6% gas mixture atmosphere of CO2 and balanced N2, as described by Trager W, et al, with some modifications. The tests for the antimalarial 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 formula14–16: % inhibition = (GRL – GRI) / GRL X 100.

The IC50 value was calculated by an activity curve: Percentage of inhibition vs. logarithm of drug concentration, through linear interpolation calculation:

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

\[X1 = \text{Concentration of the drug that gives an inhibition of } Y1 \text{ parasitemia} > 50%;\]

\[X2 = \text{Concentration of the drug giving an inhibition of } Y2 \text{ parasitemia} < 50%;\]

\[Y1 = \text{Percentage of inhibition of } X1\]

\[Y2 = \text{Percentage of inhibition of } X2\]

**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 p<0.05 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-dehydrodihydrolycorine (3), 1-O-acetylcorydine (6) with values of 19.73; 14.99; 10.36; 10.22; 10.16; 10.14; 10.04; 9.85 µg GAL/100 mg TA respectively.

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 37°C in a 5% O2, 6% gas mixture atmosphere of CO2 and balanced N2, as described by Trager W, et al, with some modifications. The tests for the antimalarial 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 formula14–16: % inhibition = (GRL – GRI) / GRL X 100.

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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 p<0.05 were considered statistically significant.
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**Figure 1:** In vitro antiplasmodial activity of the total alkaloids of the bulbs of *Clinanthus incarnatus* and *Clinanthus ruber.*

**Figure 2:** CI₅₀ 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.

<table>
<thead>
<tr>
<th>Alkaloids</th>
<th>[M⁺]</th>
<th>Rt (min)</th>
<th>RI</th>
<th>C. incarnatus</th>
<th>C. ruber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycorine-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anhydrolocrine (1)</td>
<td>251</td>
<td>23.962</td>
<td>2501.6</td>
<td>-</td>
<td>18.0</td>
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<tr>
<td>2,4-didehydro-2-dehydroxylycorine (2)</td>
<td>269</td>
<td>24.451</td>
<td>2534.4</td>
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<td>4.15</td>
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<tr>
<td>11,12-dehydroanhydrolycorine (3)</td>
<td>249</td>
<td>25.508</td>
<td>2653.2</td>
<td>10.04</td>
<td>5.81</td>
</tr>
<tr>
<td>Hippamine (4)</td>
<td>301</td>
<td>26.514</td>
<td>2705.8</td>
<td>10.16</td>
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<tr>
<td>Galanthine (5)</td>
<td>317</td>
<td>26.884</td>
<td>2730</td>
<td>10.36</td>
<td>-</td>
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<tr>
<td>1-O-Acetyllycorine (6)</td>
<td>329</td>
<td>27.142</td>
<td>2747.0</td>
<td>9.85</td>
<td>-</td>
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<tr>
<td>Lycorine (7)</td>
<td>287</td>
<td>27.693</td>
<td>2783.1</td>
<td>19.73</td>
<td>70.2</td>
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<tr>
<td>Crinine/haemanthamine-type</td>
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<td></td>
<td></td>
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<tr>
<td>Vittatine/crinine (8)</td>
<td>271</td>
<td>23.660</td>
<td>2518.7</td>
<td>10.22</td>
<td>-</td>
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<tr>
<td>3-O-acetylpowelline (9)</td>
<td>343</td>
<td>25.370</td>
<td>2630.8</td>
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<tr>
<td>8-O-demethylmaritidine (10)</td>
<td>273</td>
<td>28.327</td>
<td>2794.2</td>
<td>-</td>
<td>3.45</td>
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<tr>
<td>Galanthamine-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Galanthamine (11)</td>
<td>287</td>
<td>22.355</td>
<td>2433.2</td>
<td>14.99</td>
<td>-</td>
</tr>
<tr>
<td>Not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NI, m/z 201 ; [M=273] * (12)</td>
<td>273</td>
<td>23.904</td>
<td>2497.8</td>
<td>-</td>
<td>&lt;0.1</td>
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<tr>
<td>NI, m/z 125 (homolycorine-type)* (13)</td>
<td>125</td>
<td>29.419</td>
<td>2867.4</td>
<td>-</td>
<td>6.80</td>
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</table>

* proposed structure-type according to the fragmentation pattern; Rt: retention time; RI: Kovats Retention Index; NI: not identified.
identified: lycorine(7), anhydrolycorine (1), 11,12-dehydroanhydrolycorine(3), 2,4-didehydro-2-dehydroxylycorine(2), 8-0-dimethylmaritidine(10), hippamine (4), galanthine (5), 1-O-acetyllycorine (6), lycorine (7), vittatine(8a)/crinine(8b), 3-O-acetylpowelline (9), 8-0-demethylmaritidine (10), galanthamine (11).

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), 8-0-demethylmaritidine (10), galanthamine (11).

DISCUSSION

The amaryllidaceae family is distinguished for its exclusive alkaloids isolated from all its genera. 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. 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 elwesi[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).

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 μg/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 *Plasmodium 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 IC₅₀ 0.375 μg/ml (*C. incarnatus*) and IC₅₀ 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.

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 (IC₅₀ 1.026 μg/ml), crinine (IC₅₀ 2.110 μg/ml), haemantamine (IC₅₀ 0.703 μg/ml), 6-hydroxyhaemantamine (IC₅₀ 0.348 μg/ml), 3-epihydroxybulbispermine (IC₅₀ 1.139 μg/ml), galantamine (IC₅₀ 4.38 μg/ml), tazzetine (IC₅₀ 5.420 μg/ml), ismine (IC₅₀ > 10 μg/ml), 1-O-acetylcarotine (3.21 μg/ml), 3-O-acetylahemamine (1.14 μg/ml), bufanamine (IC₅₀ 25.9 μg/ml).

In addition, other amaryllidaceae alkaloids such as haemantidine, lycorine, 3-epihydroxybulbispermine, galantine and pancrace, also showed antimalarial activity against the chloroquine-resistant strain K1 of *P. falciparum*, with values of IC₅₀ below 1 μg/ml. 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*. 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, it was discovered that lycorine is the most powerful alkaloid against *Plasmodium falciparum*.

**CONCLUSION**

The main component of total alkaloids of the Bulbs of two Amaryllidaceae species from Northern Peru was lycorine, in addition, these species showed antiplasmodial 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.

**ACKNOWLEDGMENT**

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