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EVALUATION OF THE ANTIPLASMODIAL ACTIVITY OF VOBTUSINE ISOLATED FROM VOACANGA THOUARSII (NKAHLO WA TSOVO)

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Abstract: *Voacanga thouarsii* is a medicinal plant native to the southern region of Africa and Madagascar that occurs throughout tropical Africa, from which indolic alkaloids with various biological properties have been isolated, including antiplasmodial, antibacterial, antifungal, antitumor and antiviral activity. The aim of this study was to evaluate the antiplasmodial activity of the major compound in *V. thouarsii*. Samples of the plant collected in the district of Gondola, Manica province were used to prepare extracts by methanolic maceration from 3300g of shade-dried material, resulting in a yield of 288.9g (8.75%). The crude extract was dissolved in CH_2Cl_2, followed by extraction of the basic layer in different solvents, with yields of: Et_2O 43.4g (15.02%), 18.6g (6.43%) $CH_2Cl_{(2)}$ 4.9g (1.69%) EtOAc and n-butanol 29.3g (10.14%). The extracts from EtOAc and CH_2Cl_2 were then submitted to a chromatographic column for structural identification using mass spectrometry, UV and $^{(1)}H$ and ^{13}C NMR of the isolated compounds. The major compound isolated and characterized, with the molecular formula $C_{43}H_{50}N_4O_6$, was identified as vobtusine; a bisindolic alkaloid. The antiplamodial activity of vobtusine was tested at concentrations of 1000, 500, 100 and 10 nM in the blood phase, and the average inhibitory concentration of 500 nM was determined. The results indicate a relative activity of vobtusine against *Plasmodium falciparum* in the liver stage, showing IC_{50} at a concentration of 65 µM and in the blood stage it only shows a slight activity at a concentration of 5 µM. However, given the common use of the plant by communities for the treatment of various ailments, its medicinal interest is maintained and it is recommended that similar studies are also carried out against pathogenic microorganisms, as well as the synthesis of compounds derived from vobtusine to verify their anti-plasmodial potential in animal models.

Keywords: *Voacanga thouarsii*, vobtusine, antiplasmodial activity, antimicrobial resistance

INTRODUCTION

One of the global concerns related to resistance in the health sector is malaria. According to Mitaine-offer *et al* (2002) in many tropical and subtropical regions, malaria represents the main parasitic infection, the most severe forms being caused by *Plasmodium falciparum*. Malaria is a protozoan infection caused by parasites of the genus Plasmodium (Singh *et al*, 2022).

Although five species of Plasmodium, *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* are responsible for human malaria, severe and complicated malaria is mainly caused by *P. falciparum and P. vivax*. In addition, *P. falciparum* is responsible for the majority of malaria-related deaths worldwide, estimated at around 409,000 deaths resulting from approximately 229 million malaria cases in 2019, as reported by the WHO (Singh *et al*, 2022).

Children under the age of five and pregnant women are among the most vulnerable groups affected by malaria. Infection by malaria parasites begins clinically silently in the liver, when Plasmodium sporozoites, released by female Anopheles mosquitoes, invade hepatocytes and replicate within a parasitophorous vacuole inside the hepatic host cell. The latter provides protection as well as resources for the infectious parasite to multiply into thousands of daughter merozoites, which are finally released into the bloodstream where they infect erythrocytes, leading to the onset of the symptomatic and cyclic stage of infectious blood (Singh *et al*, 2022).

In Mozambique, malaria is the main cause of hospitalization in paediatric services (INS, 2018). In view of this, new drugs to combat malaria are needed due to the spread of *Plasmodium falciparum* resistant to available antimalarial drugs. Faced with the increasing

resistance of *P. falciparum* to traditional treatments, several research groups point to the antiplasmodial activity of alkaloids as being promising for finding new antimalarial compounds of plant origin (Mitaine-Offer, 2002).

Thus, the aim of this work is to find a new major antiplasmodial indolic alkaloid through the phytochemical study of the African medicinal species *Voacanga thouarsii*

MATERIALS AND METHODS

PLANT MATERIAL

The roots of *Voacanga Thouarsii* were collected in Mozambique, in Manica Province, in the District of Gondola, 2022

The plant material collected was dried in a ventilated place in the shade, pulverized by the grinding mill in the laboratory of the Instituto de Investigação Agrário de Moçambique and transported to the laboratory of the Faculty of Pharmacy of the University of Lisbon for further studies.

PREPARATION OF EXTRACTS

The air-dried powdered roots (3.3 kg) of *Voacanga thouarsii* were successively extracted with MeOH until 10% of the initial extraction yield was reached. The 70% methanolic extracts were prepared by maceration at room temperature and protected from light. After filtration, the solvent was distilled in a rotary evaporator at reduced pressure. Residues water were removed using Na_2SO_4 as a drying agent. The residue MeOH 288.9 g (8.75%), was dissolved in 10% Et_2O (4 X 1 L) and CH_3COOH (2 X 1L), yielding 43.4 g (15.02%), Et_2O. The pH of the acid layer was adjusted to 9 by adding dilute NH_4OH. The basic layer was extracted successively with CH_2Cl_2 (4x1L), EtOAc (4 x 1 L) and n-butanol (4 x 1 L), yielding 18.6g (6.43%) CH_2Cl_2 4.9g (1.69%) EtOAc and 29.3 g (10.14%) n-butanol soluble fractions figure 1, procedures adapted according to (Mansoor *et al*., 2013).

CHEMICAL CHARACTERIZATION OF EXTRACTS AND ISOLATION OF COMPOUNDS

The soluble fraction of EtOAc and CH_2Cl_2 was subjected to silica gel column chromatography, using solvent mixtures of increasing polarity *(n* hexane-EtOAc and EtOAc--MeOH), which resulted in fractions A-L.

The major secondary metabolites were identified and the one of greatest interest was isolated using chromatographic techniques, such as column chromatography using different stationary phases, selected according to the need for each separation.

n	Quantity (g)	*Eluents (v/v)*	
Fraction		**Hexane/EtOAc**	**EtOAc/ MeOH**
A	0,22	70/30	-
B	0,6	45/55	-
C	0.15	10/90	-
D	0.17	-	99/1
E	0.17	-	98/2
F	1.7	-	94/6
G	0.6	-	90/10
H	0.75	-	85/15
I	3.94	-	80/20
J	2.4	-	80/20
K	2.8	-	80/20
L	0.1	-	70/30

Table 1: Column chromatography of the EtOAc and Dichloromethane extract (*V. thouarsii*)

The structural identification of the majority isolated compound was carried out using Mass Spectrometry (MS), Infrared Spectroscopy (IR) and Nuclear Magnetic Resonance Spectroscopy (NMR) of ^{1}H and ^{13}C. Using these techniques, it was possible to characterize the majority compound with a mass of 3.94g, resulting from fraction I by combining EtOAc/MeOH 80:20.

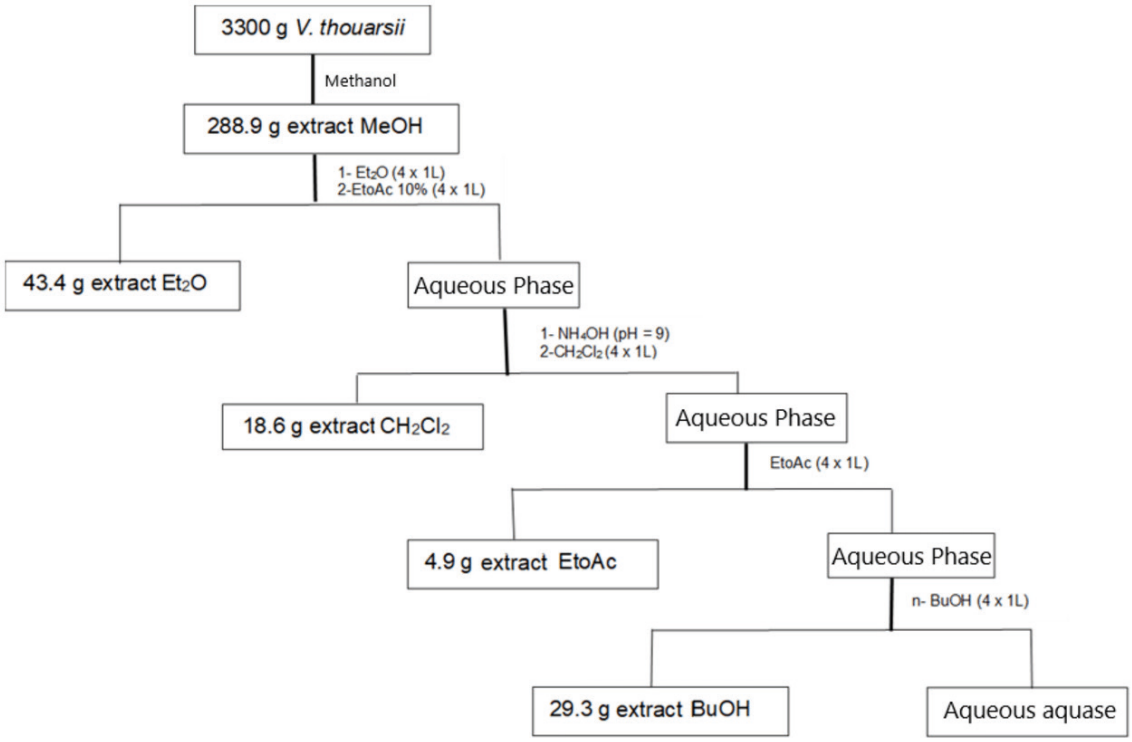
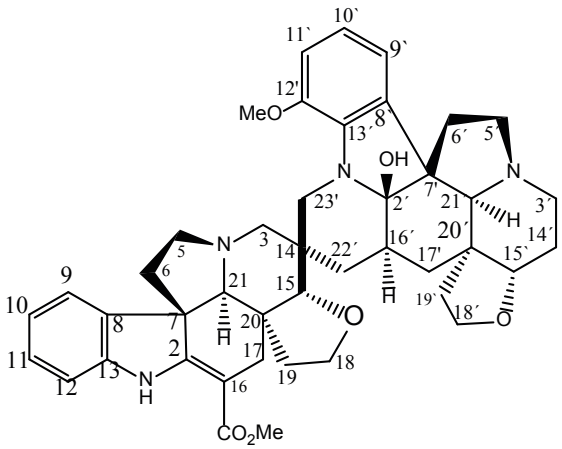


Figure 1: Preparation of fractions d the roots of *V. thouarsii according to polarity*

PHYTOCHEMICAL STUDY OF *Voacanga thouarsii*

The bioguided study of *Voacanga thouarsii* carried out in this research resulted in isolation and characterization of a major compound which is an indolic alkaloid called vobtusine according to the comparison of carbon spectrum data (^{13}C) Rolland (1976)



Vobtusine

Carbon position	1[a]	2[a]	Δδ Chemical
2	166.6	167.2	0.6
3	53.7	53.7	0.3
5	50.9	51.17	0.3
6	44.9	44.19	0.7
7	54.8	55.0	0.2
8	137.6	137.9	0.3
9	121.2	121.64	0.4
10	120.4	120.84	0.4
11	127.4	127.92	0.5
12	109.1	109.52	0.4
13	142.8	143.16	0.4
14	39.6	39.88	0.3
15	87.4	87.81	0.4
16	94.3	94.36	0.1
17	27.3	27.6	0.3
18	64.2	64.55	0.4
19	34.8	34.93	0.1
20	47.2	47.89	0.3
21	68.9	69.14	0.2
C=O	168.3	169.03	0.7
OMe	50.9	51.24	0.3

Table 2: ^{13}C votbusine NMR spectrum $CDCl_3$

Carbon position	1[a]	2[a]	Δδ
2'	93.7	94.14	0.4
3'	48.7	48.70	0.0
5'	51.9	52.14	0.2
6'	31.1	31.36	0.3
7'	55.9	56.16	0.3
8'	134.2	134.72	0.5
9'	114.5	114.92	0.4
10'	118.1	118.82	0.7
11'	110.8	111.18	0.4
12'	144.9	145.45	0.6
13'	137.2	137.42	0.3
14'	25.7	25.85	0.2
15'	80.3	80.75	0.4
16'	31.5	31.75	0.3
17'	32.4	32.59	0.2
18'	65.1	65.1	0.0
19'	36.6	36.65	0.1
20'	44.1	44.43	0.3
21'	63.6	64.02	0.6
22'	34.1	33.99	0.1
23'	46.1	46.37	0.3
Ome	55.0	55.24	0.3

Table 3: ^{13}C votbusine NMR spectrum (cont'd) $CDCl_3$

1st - Results of the carbon spectra of vobtusine (Rolland, 1976).

2nd - Experimental data results for the ^{13}C spectrum of vobtusine.

Δδ Chemical - Difference in the chemical shift of the respective carbons in two spectra.

As you can see from the chemical shift values in the two spectra in the table, in neither case is the difference greater than or equal to 1 unit, which leads us to believe that this is the same compound. On the other hand, the data from DEPT 90 and DEPT 135 spectra show that the compound under analysis has 15 methylene carbons (-CH_2-), which can be confirmed in the vobtusine structure.

According to Pavia, Lampma & Kriz (2001), in DEPT experiments carbon atoms bound to odd numbers of hydrogens appear as positive peaks, while carbon atoms bound to even numbers of hydrogens appear as negative peaks.

ANTIPLASMODIC ACTIVITY OF VOBTUSINE

ANTI-PLASMODIAL ACTIVITY

The anti-plasmodial tests were carried out at the Institute of Molecular Medicine of the University of Lisbon.

Stock solutions were prepared at 10 mM by dissolving precisely weighed compound in DMSO and stored at 20ºC or 80ºC.

IN VITRO EVALUATION OF LIVER ACTIVITY

For this case, we proceeded with the protocol described in Singh *et al* (2022), in which the activity of the compound against Huh7 cells infected *with P. berghei* was assessed *in vitro* by bioluminescence, as described above.

Briefly, Huh7 cells were seeded as indicated above the day before infection. Compound stock solutions were prepared in DMSO. Test concentrations were obtained by serial dilution of compound stock solutions in infection medium. After 1 h of incubation with dilutions of selected compounds, 1 × 104 *P. berghei* sporozoites expressing luciferase were added per well. The plates were centrifuged and incubated for 46 h at 37 °C, 5% CO_2. At this point, the impact of the compound on cell viability was assessed using the AlamarBlue assay *(Invitrogen)*, according to the manufacturer's recommendations. Next, the impact of the compounds on the parasite load was assessed by bioluminescence using the Firefy Luciferase Assay Kit 2.0 (Biotium).

EVALUATION *IN VITRO* OF THE ACTIVITY OF THE BLOOD STAGE

Synchronized ring-phase cultures of *P. falciparum* NF54 at 2.5% hematocrit and approximately 1% parasitemia were incubated with drug or DMSO (vehicle control) in 96-well plates for 48 h at 37 °C in 5% CO_2 and 5% O_2 atmosphere.

The stock solution of the compound was prepared in DMSO. The working solutions were prepared from the stock solutions in complete malaria culture medium (CMCM), consisting of RPMI 1640 supplemented with 25 mM HEPES, 2.4 mM L-glutamine, 50 µg/mL gentamicin, 0.5% w/v Albumax, 11 mM glucose, 1.47 mM hypoxanthine and 37.$_3$mM $NaHCO_3$. For each measurement, 5 µl of the culture (approximately 800,000 cells) were stained with the specific DNA dye SYBR green I at 1×. After 20 min of incubation in the dark, the stained sample was analyzed by flow cytometry using Cyfow Cube 6 (Sysmex, Germany).

Approximately 100,000 events were analyzed in each flow cytometry measurement. All samples were analyzed in triplicate and three different experiments were performed.

STATISTICAL ANALYSIS

Non-linear regression analysis using GraphPad Prism 8 (GraphPad software, La Jolla Cali fornia, USA) was employed to adjust the normalized results of the dose-response curves for IC_{50} determinations of liver and blood activities in vitro.

DETERMINATION OF CI_{50}

The IC_{50} was determined graphically as concentration versus percentage inhibition.

The compound was first tested at four different concentrations 1000 nM, 500 nM, 100 nM and 10 nM to get an idea of its activity.

From these results it was possible to choose compound concentrations to be used for IC_{50} determination.

To determine the IC_{50}, the compound was tested at seven different concentrations, comprising concentrations with zero, intermediate or total activity.

CULTURE MEDIUM - Mcm ALBUMAX

Complete Malaria Medium (MCM): RPMI 1640 supplemented with 25 mM HEPES, 2.4 mM L glutamine, 50 µg /mL gentamicin 0.5% w/v Albumax, 11 mM glucose 1.47 mM hypoxanthine and 37.3 mM $NaHCO_3$.

IN VITRO* CULTIVATION OF *Plasmodium falciparum

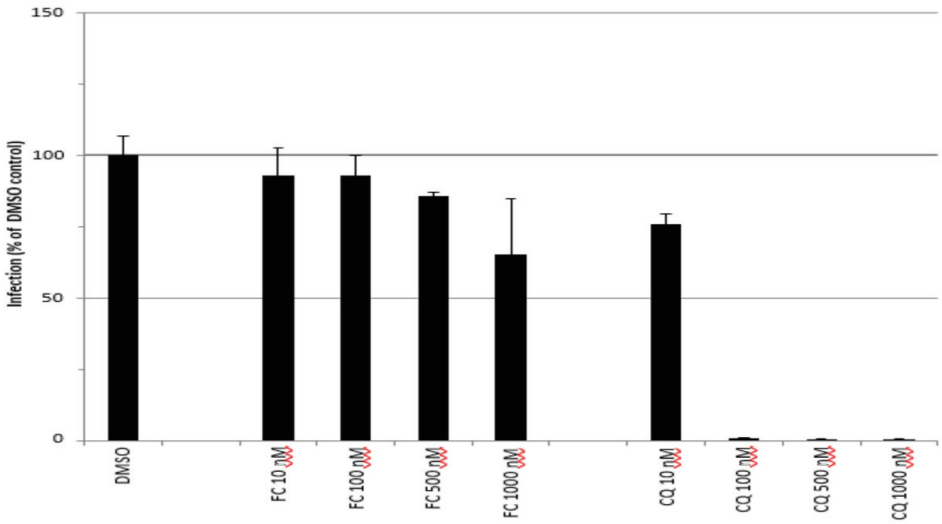
The *P. falciparum* parasites were grown and maintained in tissue culture flasks with daily changes of MCM containing 5% freshly collected human erythrocytes, under an atmosphere of 5% O_2 and 5% CO_2, at 37ºC. The level of parasitemia was assessed daily by microscopy and kept below 1% by diluting parasitized red blood cells with uninfected red blood cells. Before testing the compound, the cultures were previously synchronized in the form of a ring.

DETERMINATION OF ANTIPLASMODIAL ACTIVITY

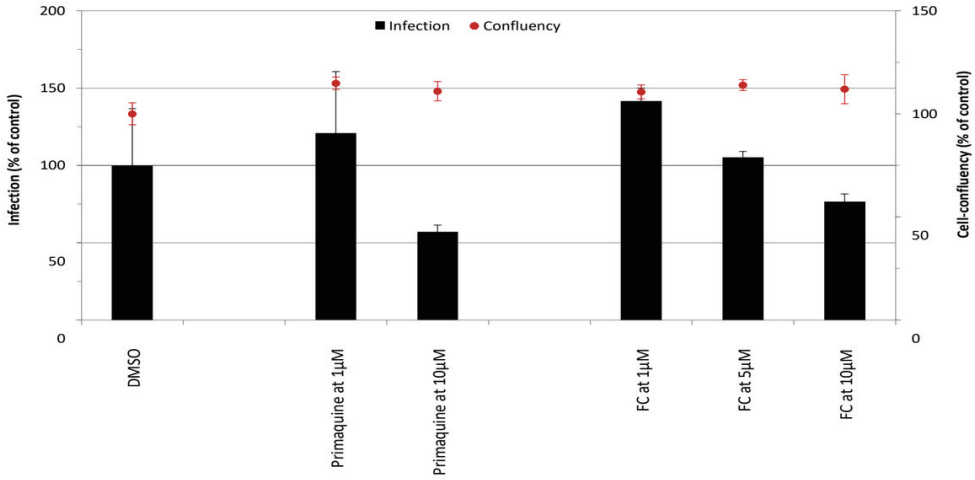
The evaluation of biological activity enabled the relative anti-plasmodial activity of vobtusine to be verified, as shown in Graph 1.

After testing the compound at four different concentrations 1000 nM, 500 nM, 100 nM and 10 nM in the blood phase, it was found that the compound showed minimal activity from 500 nM onwards, eliminating only 20% of parasites, and at 1000 nM only 40% of parasites. It should be noted that chloroquine at the same concentrations eliminates 100% of parasites, which means that pharmacologically vobtusine is inactive under ideal conditions.

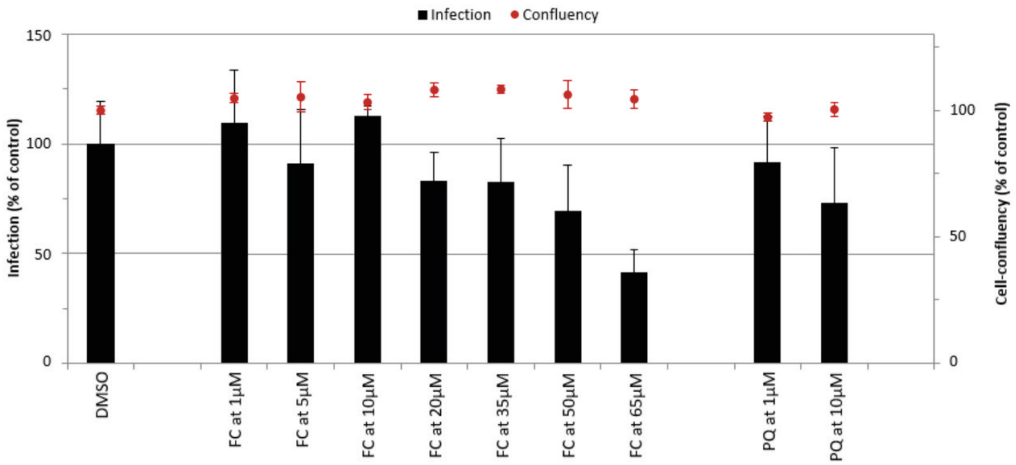
When determining the antiparasitic activity of vobtusine at three different concentrations 1 µM, 5 µM, 10 µM in the liver stage, it was found that the compound showed slight activity from 10 µM onwards, eliminating only around 20% of parasites, 25% less at the same concentration of primaquine, which is 45%. Primaquine is an alkaloid that was used as a drug to treat malaria and acts on the li-



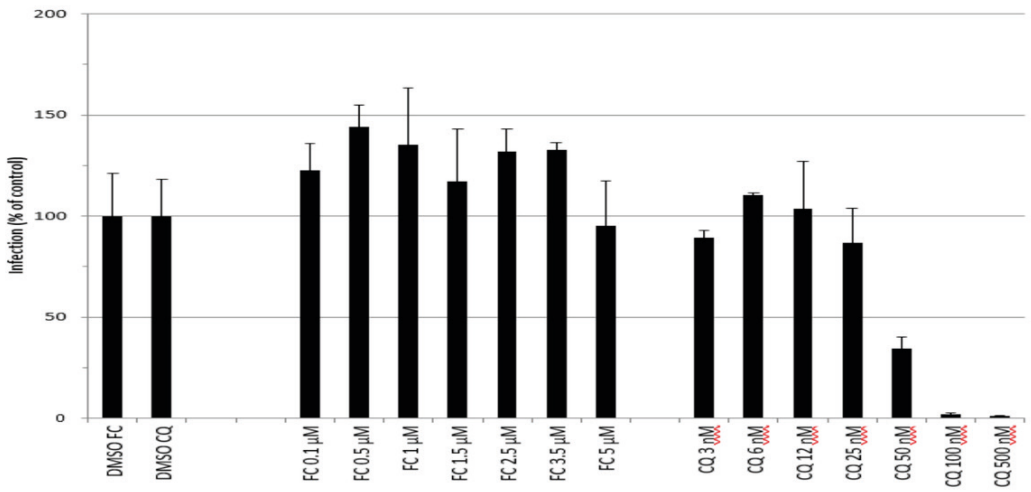
Graph 1: 1st screening of antiparasitic activity *in the in vitro* blood stage. DMSO is represented as a negative control.



Graph 2: 1st screening of liver activity in



Graph 3: Determination of IC_{50} at liver stage concentration up to 65 µM .



Graph 4: Determination of IC_{50} *in in vitro* blood stage.

ver. Looking at the data in the graph 2, it can be seen that despite its low activity, even with increasing concentrations of the compound, there is no toxicity to the cells, which is an important factor for biological tests.

The results in graph 3 show that when testing vobtusine at concentrations ranging from 1 µM to 65 µM, it was found that the compound hit over 50% of the parasites, which means that the compound only shows IC_{50} at high concentrations, which means that it has low antiplasmodial activity. However, despite these figures, the compound has an excellent confluence value, which means that up to the concentrations tested, vobtusine is not toxic to cells, which is important for future research.

It should be noted that the results of graph 6 are very reliable as can be seen in the table above by the test value of $R^2 = 0.84$.

According to the study by *Mitaine-offer et al* (2002), compounds with IC_{50} between the most active, 3.2 and 15.4 µM, and the least active, with IC_{50} between 22.6 and 52.6 µM, were considered most active. This statement makes it clear that the compound vobtusine does indeed have low activity

In this experiment carried out in the blood phase, the R^2 test is excellent since it was 0.9816, however vobtusine did not show IC_{50} when tested up to 5 µM while chloroquine had IC_{50} of 42.61% at 50 nM, which means that the indolic alkaloid in question does not show interesting antiparasitic activity in the blood phase.

CONCLUSION

According to the characterization of the ^{1}H and ^{13}C NMR and MS spectra, the major compound isolated from the roots of *Voacanga thouarsii* has the molecular formula $C_{43}H_{50}N_4O_6$ and has been identified as a bisindolic alkaloid called vobtusine.

When determining the antiparasitic activity of vobtusine at three different concentrations 1 µM, 5 µM, 10 µM in the liver stage, it was found that the compound shows slight activity from 10 µM onwards, eliminating only around 20% of parasites, 25% less at the same concentration as primaquine, which is 45%.

The results show that when testing vobtusine at concentrations ranging from 1 µM to 50 µM, the compound was only able to kill 40% of the parasites, which means that even at these concentrations it was not possible to determine the IC_{50}

When the concentrations were increased to 65 µM, it was found that the compound hit more than 50% of the parasites, so the compound only shows IC_{50} at high concentrations which means that it has low antiplasmodial activity.

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