

## ***Ocotea pulchella* as an alternative against schistosomiasis: chemical analysis, development of nanoemulsion and biological control activity**

[*Ocotea pulchella* como una alternativa ante esquistosomiasis: análisis químico, desarrollo de una nanoemulsión y actividad en control biológico]

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**Abstract:** The aim of this work was to evaluate the potential of the essential oil (EO) from *Ocotea pulchella* leaves as an alternative in the control of schistosomiasis. It was tested *O. pulchella* EO nanoformulation to assess its activity against adult *Biomphalaria glabrata*, their spawning and *Schistosoma mansoni* cercariae. Additionally, the EO chemical composition was investigated by gas-chromatography. Nanoemulsion were elaborated by the low energy method. The adult mollusks, their spawning and cercariae were placed in contact with nanoemulsion to calculate lethal concentrations. Myristicin, bicyclogermacrene and  $\alpha$ -Pinene were the main substances in the EO. Nanoemulsion caused mortality of adult *B. glabrata*, its egg embryos and *S. mansoni*. These results suggest the use of this nanoemulsion as an alternative in the control of the schistosomiasis cycle.

**Keywords:** Essential oil nanoemulsion; *Ocotea pulchella*; Mollusk control; *Biomphalaria*; *Schistosoma mansoni*; Schistosomiasis.

**Resumen:** El objetivo de este trabajo fue evaluar el potencial de los aceites esenciales (AE) de las hojas de *Ocotea pulchella* como una alternativa en el control de esquistosomiasis. Se probó una nanoformulación de AE de *O. pulchella* para evaluar su actividad ante adultos de *Biomphalaria glabrata*, sus huevos y cercarias de *Schistosoma mansoni*. La nanoemulsión fue elaborada por el método de baja energía. Los moluscos adultos, sus huevos y cercarias se colocaron en contacto con la nanoemulsión para calcular concentraciones letales. Los compuestos mayoritarios en el AE fueron miristicina, biciclogermacreno y  $\alpha$ -pineno. La nanoemulsión causó mortalidad en adultos de *B. glabrata*, sus huevos y a *S. mansoni*. Los resultados sugieren el uso de esta nanoemulsión como una alternativa en el control del ciclo de esquistosomiasis.

**Palabras clave:** Nanoemulsión de aceites esenciales; *Ocotea pulchella*; Control de moluscos; *Biomphalaria*; *Schistosoma mansoni*; Esquistosomiasis.

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## INTRODUCTION

Schistosomiasis is an acute and chronic parasitic disease caused by *Schistosoma trematodes* and transmitted by snails of various species. Nowadays, it is the second-largest infectious parasitic disease in the world after malaria. In total, 78 countries are affected, mainly in tropical and subtropical regions. In 2017, nearly 99 million people worldwide used treatment for schistosomiasis (WHO, 2018). The acute form of this disease causes symptoms such as fever, fatigue, malaise, myalgia, nonproductive cough. The later stages of this disease are characterized by abdominal pathologies such as diarrhea, hepatosplenomegaly, and diffuse abdominal pain. The chronic form of the disease occurs when *Schistosoma* lays its eggs, and host immune system reactions lead to hepatic, urinary, intestinal, and ectopic forms of the disease (Colley *et al.*, 2014).

Human schistosomiasis is caused by parasites of the genus *Schistosoma*, which use the mollusks of the genus *Biomphalaria* as intermediate hosts. In many cases, prevention methods such as eradicating these hosts using chemical pesticides are relevant to disease control. Niclosamide (Baylucide®, Bayer, Leverkusen, Germany) is the only commercially available molluscicide recommended by the World Health Organization (WHO) for large-scale use in schistosomiasis control programs (WHO, 1983). However, this substance is toxic to other organisms, and resistance to this agent makes it necessary to search for new drugs and elements to be used for intermediate host control (Inobaya *et al.*, 2014). Therefore, the WHO stimulates the search for alternative substances based on plant species (Ding-Feng, 2010). Because, plants are abundant in countries with endemic schistosomiasis and have different components in their extracts, which makes the appearance of resistance difficult (Tavares *et al.*, 2007).

Many plant extracts and oils exhibiting intrinsic molluscicidal activity may have problems with solubility in the aquatic environment due to the low polarity. In this context, nanotechnology has been used to circumvent this problem and promote the stability of active substances. There are currently several drug nanocarriers, including nanoparticles, nanoemulsions (NEs), and liposomes (Donsi & Ferrar, 2016).

Nanoemulsions have been widely used as nanocarriers of essential oils and hydrophobic substances. NEs are dispersions of nanometric oil droplets (20-200 nm) in water, stabilized by

surfactants (Sagalowicz & Leser, 2010; Jaiswal *et al.*, 2015). Some key advantages of these nanocarriers are their easy preparation, simple composition, low production cost, industrial production possibility, and high thermodynamic stability (Tromer & Neubert, 2006; Campos *et al.*, 2012).

The species *Ocotea pulchella* is popularly known as "canelinha", "canela-preta", "canelalageana" (Marques, 2001), and "inhumirin" (Quinet *et al.*, 2015). In Brazil, it is distributed in the North, Midwest, Southwest and South regions (Quinet *et al.*, 2015). Although botanical and agronomic studies are found for this species, little information is known about its chemical composition and biological importance. On the other hand, other species of the genus *Ocotea* have the chemical description of essential oils, which in turn have large compounds with molluscicidal activity (Rambo, 2014; Leite *et al.*, 2009). Thus, the aim of this work was to investigate the chemical composition of the essential oil from *O. pulchella* leaves and their activity in the control of schistosomiasis.

## MATERIALS AND METHODS

### *Plant material*

Fresh leaves from three specimens of *Ocotea pulchella* were collected from Restinga de Jurubatiba National Park, at the coordinates 22°12'697" S - 41°35'321" W, 22 ° 12 688"S - 41 ° 35'324 "W, and 22 ° 12'692" S - 41 ° 35'331 "W. This work was realized under the authorization number 13659-12 (SISBIO) and A0D648D (SISGEN). The species was identified by the botanist Dr. Marcelo Guerra Santos, and a voucher sample was deposited in the herbarium of the Faculty of Teacher Education, under the number 16451 (RFFP) (Rio de Janeiro State University, Brazil).

### *Essential oil extraction*

Leaves of *O. pulchella* (1810.9 g) were turbolized with distilled water. Then, the material was placed in three 5 L round bottom flask and hydrodistilled for 4 h in a Clevenger type apparatus. The essential oil obtained was stored at 4°C for further chemical analysis and development of NEs.

### *Gas chromatography analysis*

Gas chromatography (GC) was performed under the following conditions: injector temperature, 260°C; GC/MS detector, 290°C; carrier gas, Helium; flow rate, 1 mL/min; Split, 1:40. The oven temperature was initially 60°C and then rose to 290°C at a rate of

3°C/ min. One microliter of each sample dissolved in dichloromethane (1:100 mg/ mL) was injected into a DB-5 column (0.25 µm x 30 m x 0.25 µm). Electron ionization by mass spectrometry (MS) was 70 eV, and the scanning speed was 1 Scan/ s. Retention rates (RI) were calculated by extrapolating the retention times of an aliphatic hydrocarbon mixture (C9-C30) analyzed under the same conditions (van der Dool & Kratz, 1963).

Substances were identified by comparing their arithmetic indices (AI) and mass spectra with those reported in the literature (Adams, 2007). The MS fragmentation pattern of the compounds was also compared to the NIST mass spectrum libraries (database program containing mass spectra of over 100,000 substances). Quantitative analysis of the chemical constituents was performed by flame ionisation gas chromatography (CG/FID), under the same conditions as GC/MS analysis. Percentages of these compounds were obtained by FID peak area normalisation method.

#### ***Nanoemulsification method and determination of hydrophilic-lipophilic balance (HLB)***

Emulsification was produced by modifying the low energy method described by Ostertag *et al.* (2012). The emulsions were composed of 5% (w/w) *O. pulchella* oil, 5% (w/w) surfactants and 90% (w/w) water. Span 80 and Tween 20 were used as surfactants to prepare NEs. Several emulsions with HLB values ranging from 4.3 to 16.7 were prepared by mixing the surfactants in different proportions. For the preparation of the NEs, the oily phase consisting of *O. pulchella* oil and surfactants was homogenized by magnetic stirring (400 rpm) for 30 min at room temperature. After that, the aqueous phase (distilled water) was added to the magnetic stirring oil phase (400 rpm) for an additional 1 h. The formulations were analyzed for stability by droplet size analysis and polydispersion index (PDI) values. The formulation with the smallest droplet size and PDI values indicated the appropriate oil HLB.

The droplet size and polydispersity index (PDI) of the NEs were determined by photon correlation spectroscopy (Zetasizer ZS, Malvern, UK). Droplet measurements were performed in triplicate, and the mean droplet size was expressed as mean diameter ± standard deviation.

#### ***Molluscicidal Assays***

The action of the selected NE was evaluated for biological control of *B. glabrata* using the

methodology of Santos *et al.* (2017). Clams of size 10-12 mm were individually placed in 24-well plates and exposed to concentrations of 20, 40, 60, 80, 100 and 120 ppm in the final 2 mL NE volume. The mortality of the snail was compared with the negative controls: NE white (containing only distilled water and surfactants - 2 mL) and distilled water (2 mL), and with the positive control: niclosamide (2 mg/L, 2 mL). Mortality was assessed at 24 h and 48 h, and the absence of shell retraction and hemolymph release were the criteria to evaluate mortality.

#### ***Evaluation of ovicidal activity***

On the initial day, the Styrofoam plates were deposited in the water of the *B. glabrata* rearing tanks for oviposition. After 48 h, the egg capsules were carefully removed from the Styrofoam and then accommodated in 24-well plates using the adapted methodology of Araújo *et al.* (2019). Then, the number of viable eggs at time zero was counted, and then 1000 µL of NE was added to the plate wells at concentrations of 20, 40, 60, 80, 100 and 120 ppm. After 24 h and 48 h of exposure, viable egg counts were repeated.

#### ***Evaluation of cercarial activity***

In a 24-well plate, the amount of *S. mansoni* cercariae present in 1000 µL was initially estimated using 20 µL Lugol and counted in a stereomicroscope. Then, in another well of the plate, 1000 µL of *S. mansoni* cercaria suspension and 1000 µL of NE at 20, 40, 60, 80, 100, and 120 ppm were added. After that, 20 µL of 0.1% Trypan Blue dye was added. Dead blue cercariae were counted at 1 h, 2 h, 3 h, and 4 h.

#### ***Statistical analysis***

Statistical analysis of the experiments was performed using the Prism 6 GraphPad program (GraphPad software), using one-way ANOVA, two-way ANOVA, followed by the Tukey test with a  $p < 0.001$ . Linear regression with  $p < 0.0001$  and  $R^2 = 0.9647$  were used as significant statistical parameters.

## **RESULTS**

### ***Essential oil***

The essential oil obtained from fresh leaves showed a translucent and transparent appearance with a volume of 19 mL and yielded 1.05%. In total, it was identified 20 substances (Table No. 1). There is a predominance of monoterpene hydrocarbons (41.87%), followed by 31.45% phenylpropanoid and 24.29% of hydrocarbon sesquiterpene. The main

substances were myristicin (26.34%),  $\alpha$ -pinene (17.20%), and bicyclogermacrene (16.57%).

**Table No. 1**  
**Chemical composition of the essential oil of *Ocotea pulchella* (Mart.) leaves**

AI LIT	AI EXP	SUBSTANCES	%
932	935	$\alpha$ -pinene	17.2
946	949	canphene	0.3
974	979	$\beta$ -pinene	8.3
988	992	myrcene	1.2
1002	1008	$\delta$ -carene	9.5
1020	1026	cymene	2.3
1024	1030	limonene	0.9
1026	1032	cyneol	0.5
1086	1091	terpinolene	2.2
1335	1339	$\delta$ -elemene	0.6
1374	1378	$\alpha$ -copaene	0.3
1389	1395	$\beta$ -elemene	0.6
1417	1423	$\beta$ -caryophyllene	1.6
1492	1485	$\delta$ -selinene	2.0
1500	1503	bicyclogermacrene	16.6
1517	1508	myristicin	26.3
1522	1528	$\delta$ -cadinene	0.3
1568	1582	$\beta$ -isoelemicine	5.1
1577	1586	spathulene	2.3
2042	2046	kaurene	1.9
		Total Identified	100.0
		Monoterpenes hydrocarbons	41.9
		Phenylpropanoid	31.4
		Sesquiterpenes hydrocarbons	24,3
		Diterpene	1.9
		Oxygenated Monoterpenes	0.5

### **Nanoemulsion**

It was prepared 11 formulations with HLB values ranging from 16.7 to 4.3 (Table No. 2). Then the six formulations with the best HLB indices, which ranged from 10.5 to 13, were refined. The formulation selected presents an HLB value of 11.5, droplet size of 85.42 nm and PDI value of 0.284 (Table No. 3).

### **Molluscicidal assay**

The evaluation of molluscicidal activity was

performed to determine the lethality of the NE of the essential oil of *Ocotea pulchella* leaves in the schistosomiasis *B. glabrata* transmitting species. In the molluscicidal assay, the LC<sub>50</sub> and LC<sub>90</sub> values over the 24h period were LC<sub>50</sub> = 45.78 ppm and LC<sub>90</sub> = 64.14 ppm, while in the 48h period were LC<sub>50</sub> = 38.58 ppm and LC<sub>90</sub> = 42.97 ppm (Figure No. 1).

### **Ovicidal assay**

It was evaluated the action of NE in the ovicidal assay, within 24h and 48h. It was observed LC<sub>50</sub> =

33.53 ppm and  $LC_{90} = 39.25$  ppm in 24h. At 48h, all tested concentrations of NE led the eggs to death

(Figure No. 2).

**Table No. 2**  
EHL values of different Nanoemulsion formulations from *Ocotea pulchella* and their respective percent composition

Formulation	EHL	TWEEN 20	Span 80	Water
1	16.70	0.250	0	4.5
2	15.46	0.225	0.25	4.5
3	14.22	0.200	0.50	4.5
4	12.98	0.175	0.75	4.5
5	11.74	0.150	0.100	4.5
6	10.50	0.125	0.125	4.5
7	9.26	0.100	0.150	4.5
8	8.2	0.75	0.175	4.5
9	6.78	0.50	0.200	4.5
10	5.54	0.25	0.225	4.5
11	4.30	0.0	0.250	4.5

**Table No. 3**  
Droplet size, PDI and HLB values of the six *Ocotea pulchella* essential oil formulations

Formulation	Droplet size (nm)	PDI	EHL
F1	97.85	0.193	10,5
F2	99.85	0.207	11.0
F3	85.50	0.284	11.5
F4	87.09	0.282	12.0
F5	160.20	0.751	12.5
F6	93.43	0.295	13.0

#### **Cercaricidal activity**

The results of the cercaricidal activity of the NE during the 4h period were:  $LC_{50}/1h = 74.27$  ppm,  $LC_{90}/1h = 121.79$  ppm;  $LC_{50}/2h = 57.96$  ppm,

$LC_{90}/2h = 98.12$  ppm;  $LC_{50}/3h = 48.87$  ppm,  $LC_{90}/3h = 89.01$  ppm and  $LC_{50}/4h = 44.89$  ppm,  $LC_{90}/4h = 86.01$  ppm (Table No. 4 and Figure No. 3).

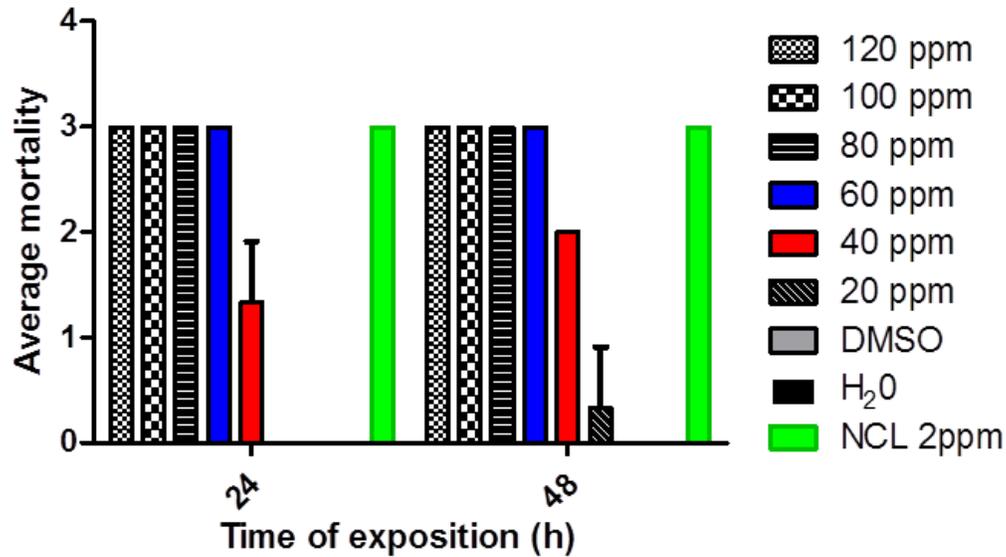


Figure No. 1

Dose-response relationship between mortality (percentage) of *Biomphalaria glabrata* (n = 3) and *Ocotea pulchella* nanoemulsion. This experiment was performed in triplicate on at least three separate days. Adult molluscicide test with *Biomphalaria glabrata* within 48 h of nanoformulation exposure. NCL - Niclosamide, DMSO-Dimethyl Sulfoxide

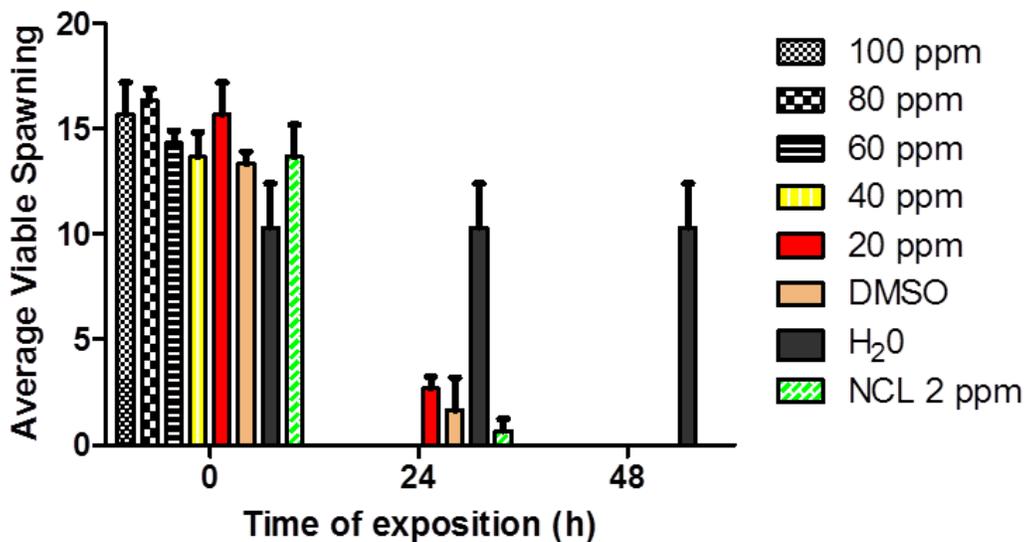
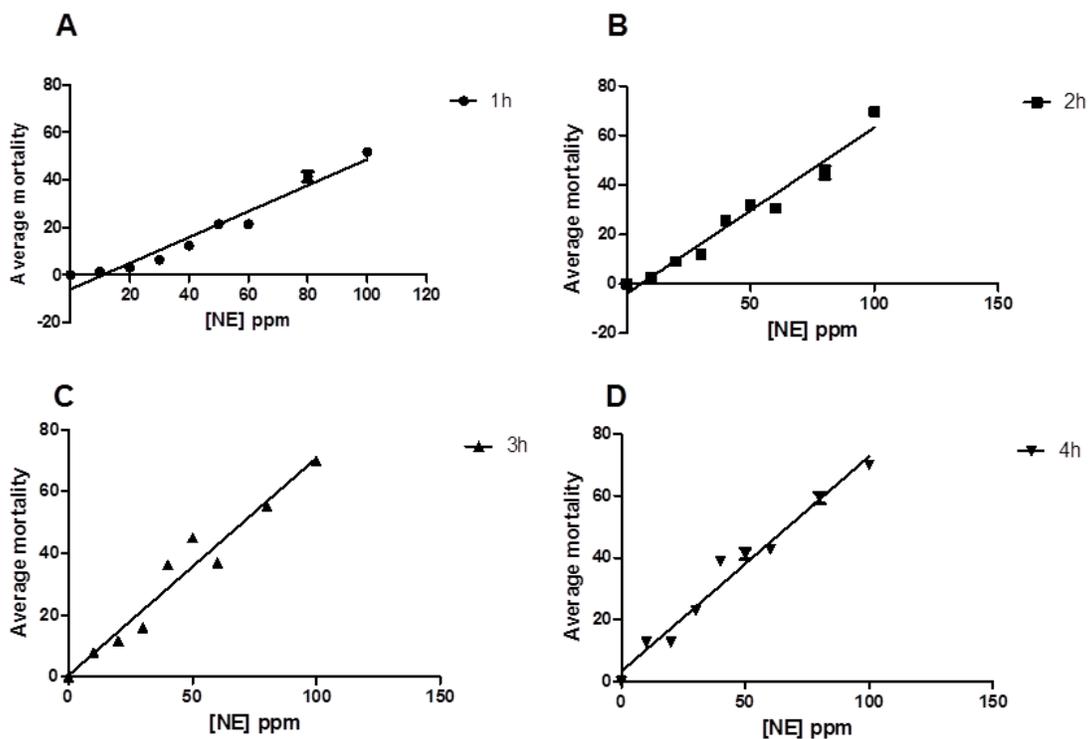


Figure No. 2

Concentration-response relationship between average viable spawning of *Biomphalaria glabrata* embryos and *Ocotea pulchella* nanoemulsion for 48 h of exposure. The results expressed in the graph represent the mean ± standard error. NCL - Niclosamide®, DMSO-Dimethyl Sulfoxide.  $p < 0.05$

**Table No. 4**  
**Lethal nanoemulsion concentrations (LC<sub>50</sub> and LC<sub>90</sub>) at 1 to 4 h exposure periods**

Time of Exposition	LC <sub>50</sub>	LC <sub>90</sub>
1 h	74.27 ppm	121.79 ppm
2 h	57.96 ppm	98.12 ppm
3 h	48.87 ppm	89.1 ppm
4 h	44.89 ppm	86.1 ppm



**Figure No. 3**

Concentration-response relationship between average mortality of *Schistosoma mansoni* cercariae and *Ocotea pulchella* nanoemulsion exposed for 1 h (A), 2 h (B), 3 h (C), and 4 h (D). The results expressed in the graph represent the mean ± standard error

## DISCUSSION

Currently, the control of schistosomiasis includes several methods such as prevention, population control of vector mollusks, and the use of antiparasitic agents in the treatment of the disease (WHO, 2016). The control of intermediate hosts of parasites of the genus *Schistosoma*, such as *Biomphalaria glabrata*, is a widely used method, but it is dependent on the few commercially available molluscicidal agents, which also have restrictions, such as the occurrence of biological resistance to these agents, as well as their effects environmental impacts, negatively affecting the local ecosystem (Inobaya *et al.*, 2014). Besides, the high risks of side effects and increased antiparasitic resistance also become sufficient reasons for the search for new agents that act on parasites in their various forms (Catanhede *et al.*, 2010). Therefore, studies involving the search for new drugs with molluscicidal and/ or antiparasitic potential against *Schistosoma* are necessary. Preferentially, studies involving plant derivatives aiming at the following aspects: effectiveness, economic viability, low environmental impact, and generation of new technologies in the fight against schistosomiasis (Brasil, 2014; Nascimento *et al.*, 2018).

The genus *Ocotea* is described in the literature about its broad pharmacological and biological activities, such as insecticide, larvicide, and anticholinesterase (Yamaguchi *et al.*, 2012; Mossi *et al.*, 2013; Scalvenzi *et al.*, 2019). However, this study is a pioneer in the research of biological activity with *Ocotea pulchella* essential oil. The phytochemical analysis identified Myristicin,  $\alpha$ -Pinene, and Bicyclogermacrene as the three main essential oil substances in *O. pulchella* leaves. Bicyclogermacrene and  $\alpha$ -pinene were also found in other species of the genus between the two main essential oil substances, such as *Ocotea indecora*, *Ocotea caudata*, and *Ocotea morae*, while myristicin was first reported as the main substance in the essential oil of leaves of the genus *Ocotea* (Prieto *et al.*, 2010; Chaverri *et al.*, 2011; Rambo, 2014; Gil *et al.*, 2016).

Myristicin (26.34%),  $\alpha$ -pinene (17.20%), and bicyclogermacrene (16.57%) are the major components of the *O. pulchella* essential oil from leaves. These substances already have been described by having biological and pharmacological activities. The compound myristicin demonstrated insecticidal and acaricidal activity (Lichtenstein & Casida, 1963), monoaminoxidase inhibition (Truitt *et al.*, 1963), and

anticholinergic effect (McKenna *et al.*, 2004). The monoterpene  $\alpha$ -pinene showed nociceptive (Him *et al.*, 2008), anti-inflammatory and chondroprotective activities (Rufino *et al.*, 2014), as well as antifungal against *Candida albicans* and *Cryptococcus neoformans*, antibacterial against penicillin-resistant *Staphylococcus aureus* (Rivas da Silva *et al.*, 2012) and molluscicide activity (Leite *et al.*, 2009). This monoterpene also showed an anticholinesterase activity of  $IC_{50} = 0.96$  mM (Zarrad *et al.*, 2017). The substance bicyclogermacrene also demonstrated molluscicide activity against three different species of *Biomphalaria*, including *B. glabrata* (Araujo *et al.*, 2019), and antifungal activity (Silva *et al.*, 2007). These findings corroborate with the results found in this work and suggest a molluscicide activity against *B. glabrata*.

The NEs prepared in this study by modification of the low energy method of Ostertag *et al.* (2012), allowed better control of droplet size in the preparation of emulsions. The extremely low particle size provided greater resistance to the effects of cremation and sedimentation, presenting a lower interfacial tension. In addition, this system allows easier solubilization of the different substances (Salager *et al.*, 2001; Solans *et al.*, 2005).

The average particle size and polydispersion index values, besides the visual observations, were the parameters of physicochemical evaluation to choose the best proportion of surfactants in the NE. Results were obtained with the Nano SZS particle test. 90 (Malvern Instruments, Worcestershire, United Kingdom). The biodegradability of the formulation is another factor that minimizes the residual toxicity of molluscicidal agents to the environment and can be used to compare the mortality rate among *Biomphalaria* populations, optimizing the resistance to NE effect, which is not the case with Niclosamide (Mishra *et al.*, 2018). Currently, the use of this product is effective, however, it is included in grade III chemical toxicity, killing both snails and eggs, as well as local faunas and flora. Also, *Biomphalaria* can shrink, bury itself in the substrate and still move away from the area of the highest concentration of dissolved products in water, even if it is in the soil (Oliveira-Filho, 2003).

NE is a product of operational simplicity, has been shown to be effective, has low reagent costs, has good dispersion in the aquatic environment, which makes this formulation have advantages over the use of Niclosamide. These objectives are guided by the schistosomiasis control program (PCE), 2008, in

which alternative research methods are suggested in plants with molluscicidal activities, confirming the need for efficient and ecologically acceptable molluscicides (Brasil, 2014).

NE molluscicidal activity showed  $LC_{50} = 45.78$  ppm and  $LC_{90} = 64.14$  ppm after 24h, which classifies a plant extract as active when it has an  $LC_{90}/24h$  value below 100 ppm (WHO, 1983).

In the cercariae activity assay, the NE presented  $LC_{90} = 86.69$  ppm within the 4h period, proving to be effective when compared to the control group. The data obtained in this work demonstrated experimental similarity with the study by Albagouri et al. (2014). In their work, they presented results of different Sudanese plant species with cercaricidal activity showing  $LC_{50}$  and  $LC_{90}$  values below 100 ppm, corroborating with the results found in the present work.

Evaluations of the 24h period showed that 40 to 100 ppm concentrations led to 100% of the eggs to death, and the 20 ppm concentration obtained high but not total embryo mortality. In 48h, even the concentration of 20 ppm could kill 100% of the eggs. Watanabe (1997) reports the great importance of embryonic stage testing in *Biomphalaria* sp. serving as bioindicators in polluted waters and in tests performed as biomarkers in testing and mutagenicity.

The present study demonstrated the molluscicidal activity of the nanoemulsified essential oil of *O. pulchella* leaves in embryos and adult forms of schistosomiasis transmitters *B. glabrata*, as well as the cercaricidal activity. Thus, the biotechnological product developed from *O. pulchella* exhibited action at different stages of the schistosomiasis cycle, being

a promising alternative in the control of this disease.

## CONCLUSION

The chemical analysis of the essential oil from *Ocotea pulchella* leaves was performed for the first time, presenting  $\alpha$ -pinene, bicyclogermacrene, and myristicin as main constituents. Therefore, it was described the effect of *O. pulchella* essential oil nanoemulsion on the control of neglected tropical and subtropical diseases, specifically against the species *Biomphalaria glabrata* and its embryos, both involved in *Schistosoma mansoni* schistosomiasis and cercariae transmission etiological agent of this disease. Thus, these results suggest the use of this product as a promising alternative for controlling the life cycle of *B. glabrata* species, the main vectors of schistosomiasis, and against its etiological agents, *S. mansoni*.

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