**Abstract:**

**Background:** The application of natural substances with specific biological actions are targets of research aimed at a more sustainable production, implying several sources of substances for new therapeutic formulations. This study presents the chemical characterization, toxicity, antimicrobial activity, larvicidal and molluscicidal activivty of the essential oil (EO) of the leaves of *Bixa orellana* Labill. **Methods:** Chemical characterization was obtained by GC/MS. The toxicity assay was performed through the bioassay with *Artemia salina* Leach, *Aedes aegypti* e *Biomphalaria glabarata*. The methods of Disc Diffusion and Dilution in Broth were used to evaluate antimicrobial activity in the face of bacteria. **Results:** LC50 in the toxicity assay was checked and classified EO as non-toxic. The EO showed antimicrobial activity against the microorganisms tested, LC50 19.49 and LC90 54.15 mg L-1 against the larvae of *Aedes aegypti*, and LC50 17.33 and LC90 39.45 mg L-1. **Conclusions:** The results obtained highlight the importance of pointing out this product as a therapeutic alternative, encouraging its application potential.

**Keywords:** essential oil*; Bixa orellana* L.; larvicidal; moluscicide.

1. **Background**

Medicinal plants are defined as those capable of producing active principles that can alter the functioning of organs and systems, restoring a state of balance or homeostasis to the organism in cases of diseases, and that can serve as precursors of semisynthetic drugs. The healing power of vegetables has been known since ancient times and has been constantly used by the pharmaceutical industry [1].

Brazil tops the list of the richest countries in biodiversity in the world, which implies several sources of substances for therapeutic formulations, including about 55,000 species and only 25% of the registered herbal medicines come from plant species present in South America [2], factors that attract the attention of health care programs and researchers worldwide, due to their medicinal and organoleptic properties [3].

Much of what is known today about plant treatments comes from popular knowledge. Despite the evolution of scientific knowledge, the use of plants as an alternative method of cure is still very frequent, a fact that occurred mainly due to and the ease of obtaining them the high cost of synthetic drugs [4].

The properties of these medicinal plants are directly related to their essential oils (EOs), which according to [5] and [6] are components that integrate the secondary metabolites of plants, that is to say, they are part of the non-primary system of these organisms, having protective functions against elements external to them.

EO is a natural product derived from medicinal plants, which have potential in the control of diseases in plants, because they have antifungal, antibacterial and insecticidal characteristics, besides presenting low toxicity to the environment and to humans [7]. In addition, they are used in the pharmaceutical and perfumery industry, in the manufacture of hygiene and cleaning products [8], besides being able to be incorporated into the formulation of synthetic products, in order to reduce or even replace the use of toxic elements [9].

In this sense, studies with natural products have great significance, especially linked to the occurrence of diseases caused by the Mosquito *Aedes aegypti,* which has grown rapidly. This growth accounts for the great relevance of research on new methodologies for combating larvae of the mosquito. Thus, because they present lower toxicity, essential oils (EOs) are important alternatives for solving this problem [10].

Among plants with medicinal properties, annatto stands out, also known by its scientific name *Bixa orellana* Labil, a plant native to the tropical region of America [11]. Its application occurs both in industry and in the popular use as food and textile dye and for pharmacological purposes, since it has antimicrobial, antioxidant, diuretic, antifungal, antileishmanial activity, among others [12,13, 14]. Popularly known as “urucum” in portuguese, a word derived from the native language of the "Guaraní (ru-ku)", meaning red, the "annatto" was the first vegetable dye to be marketed in large quantities to Europe [15]. Its seeds consist of substances such as cellulose (40 to 45%), sugars (3.5 to 5.2%), EOs (0.3 to 0.9%), proteins (13 to 16%), alpha pigments and beta carotenes (4.5 to 5.5%), and compounds such as tannins and saponins [16].

The dye extracted from *B. orellana* is considered harmless and its toxicity is practically nil, not only after ingestion but after contact with the skin [17].

In addition, the plant is also used in wounds, bruises, burns, sore throats and in diseases such as bronchitis and asthma, because it has anti-inflammatory and cicatrizing activity [18]. Thus, this study reports for the first time the toxicity, antimicrobial, larvicidal and antioxidant activities, chemical characterization and total phenolic compounds of The EO of *B. orellana* L. leaves in order to deepen [the] knowledge regarding their medicinal characteristics and properties making available the knowledge to the population about possible contraindications and emphasize some warnings and precautions to be taken.

**2. Methods**

*2.1. Botanical material*

The collection of the plant material used in this research was carried out in October 2019. The leaves of *B. orellana* (annatto) were collected in the Herbário Ático Seabra, available to the public, of the Universidade Federal do Maranhão (UFMA) and a sample deposited under record no. 00815. The formal identification of the species was performed by Prof. Dr. Ana Zélia Silva of the Departamento de Farmácia, Universidade Federal do Maranhão (UFMA). After collection, the plant material was transported to the Laboratory of Research and Application of Essential Oils (LOEPAV/UFMA), where it was submitted to the kiln of convective air drying FANEM 520 to 45ºC for 24 hours, and later crushed in knife mill.

*2.2. Essential oil*

For extraction of the EO, the hydrodistillation technique was used with a glass Clevenger extractor coupled to a round-bottomed balloon packed in an electric blanket as a heat generating source. 90g of the dried leaves of *B. orellana* were used, adding distilled water (1:10). Hydrodistillation was conducted at 100°C for 3h collecting the extracted EO. Each EO was dried by percolation with anhydrous sodium sulfate (Na2SO4) and centrifugate. These operations were carried out in triplicates and samples stored in amber glass ampoules under 4°C cooling. Subsequently submitted the analyses. The physicochemical parameters of the EOs were determined: density, solubility, color and appearance according to the [17].

*2.3. Chemical constituents*

The EO constituents were identified by gas chromatography coupled to mass spectrometry (GC-MS) at the Catalysis, Fuels and Environmental Center of the Federal University of Maranhão (NCCA-UFMA). 1.0 mg of the sample was dissolved in 1000 μL of dichloromethane (purity 99.9%). The conditions of analysis were as follows: Method: Adams. M; Injected volume: 0.3 μL; Column: Capillary HP-5MS (5% diphenyl, 95% dimethyl polysiloxane ) (Equivalent DB-5MS or CP-Sil 8CB LB/MS), in dimensions (30 m x 0.25 mm x 0.25 μm); Drag gas : He (99.9995); 1.0 mL/min; Injector : 280 oC, Split mode (1:10); Oven: 40 oC (5.0 min.) up to 240 oC at a rate of 4 oC .min-1, from 240 oC to 300 oC (7.5 min) at a rate of 8 oC.min-1 ); tT = 60.0 min; Detector : EM1; EI (70 eV); Scan mode (0.5 sec/scan); Mass range: 40 - 500 daltons (one); Line transfer: 280 oC.; Filament: off 0.0 to 4.0 min; Linear quadrupole mass spectrometer. The AMDIS (Automated Mass spectral Deconvolution Mass & Identification System) program was used to identify the compounds in the sample.

*2.4. Total phenolics*

The determination of total phenolic compounds of the EO was performed with adaptation of the Folin-Ciocalteu [19]. 5 mg of the essential oil diluted in 1 mL of ethanol was used. To this solution was added 3 mL of distilled water, 500 μL of Folin-Ciocalteu reagent and 2.0 mL of sodium carbonate at 20%. The solution formed was taken to the water bath at 50 ºC for 5 min, removed and left to cool; and then, the reading was performed in a manual spectrophotometer, in a length of 760 nm. The readings were performed in a spectrophotometer at 760 nm, and the standard curve expressed in mg of tannic acid.

*2.5. Toxicity*

This test was performed according to the methodology described by [20]. In a rectangular container, with a partition containing holes of approximately 0.02 cm thickness spaced by 0.5 cm and evenly distributed, artificial saline solution (60 g L-1 of distilled water) were added (60 g of sea salt/ 1L of distilled water). The container was placed inside an incubator illuminated by a fluorescent lamp, with aeration. On one side of this container, about 64 mg of *Artemia salina* cysts were added, taking care that they did not cross the partition. The part of the system containing artemia saline cysts was covered with aluminum foil, so that the organisms, at birth, were attracted by light on the other side of the system, forcing them to cross the partition. This procedure aims at homogenizing the physical conditions of the test organisms. Incubation was performed for a period of 48h. Throughout the test the temperature was monitored.

For the evaluation of the lethality of *Artemia salina* Leach, a saline solution was prepared stock of each EO in the concentration of 10.000 mg L-1 and 0.02 mg of Tween 80 (active tense). Rates of 5, 50 and 500 μL of this were transferred to test tubes and supplemented with saline solution previously prepared up to 5 mL, obtaining at the end concentrations of 10, 100 and 1000 mg L-1, respectively. All tests were carried out in triplicates, where ten larvae in the nauplium phase were transferred to each of the test tubes.

A solution of 5 mL of NaCl 30 g L-1 was used for white. For positive control, a solution of K2Cr2O7 and for negative control 5 mL of a solution of 4 mg L-1 of Tween 80 (active tense) were used. After 24 hours of exposure, the count of the live larvae was performed, considering dead those that did not move during observation or with the slight agitation of the vial. The criterion established by [36] was adopted for classification of EOs toxicity, being considered highly toxic when LC50 ≤ 80 mg L-1, moderately toxic to 80 mg L-1 ≤ LC50 ≥ 250 mg L-1 and slightly toxic or nontoxic when LC50 ≥ 250 mg L-1. Statistical analysis of the data for the toxicity test was performed according to the [21].

*4.6. Standardization of microbial inoculum for sensitivity tests*

Four strains of bacteria were used: *Escherichia coli* (ATCC ® 25922™) and *Staphylococcus aureus* (ATCC® 25923™), *Pseudomonas aeruginosa* (ATCC ® 15442™) and *Salmonella* sp. (ATCC ® 700623™). These were previously identified and confirmed by biochemical tests. Pure microbial cultures maintained in TSA Agar were peaked for brain and Heart Infusion Broth (HIB) and incubated at 35 °C until they reached exponential growth phase (4-6 h). After this period, the cultures had their cell density adjusted in 0.85% sterile saline solution, in order to obtain turbidity comparable to that of the standard McFarland solution 0.5, which results in a microbial suspension containing approximately 1.5x108 CFU mL-1 according to [22].

*2.7. Disk Diffusion Method (DDM)*

The Disc Diffusion technique was performed according to the [22] which standardizes the sensitivity tests of antimicrobials by disc-diffusion. First, the plates were prepared with the Mueller Hinton Agar (MHA) culture medium after its solidification was distributed to the microbial suspension on the surface of the agar and left at room temperature for 30 min. Soon after the discs containing 50 μL of EOs and discs with defined concentrations of antibiotics. Using sterile tweezers, the discs were distributed on the surface of the agar. The positive control Gentamicin (30 μg) was used. The plates were incubated in a bacteriological greenhouse at 35 °C for 24 hours. The diameters of the inhibition halos were measured, including the diameter of the disc. These trials were done in triplicate. The values of the inhibition halos were the mean measurements of the three results. Tests performed in triplicate.

*2.8. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

The Minimum Inhibitory Concentration (MIC) assay was performed using the broth dilution technique, proposed by the [22]. First, 2% solutions were prepared using dimethylsufoxide (DMSO) at 2%, and serial dilutions were prepared in MH Broth, resulting in concentrations of 10 to 1000 μg mL-1. Microbial suspension containing 1.5 x108 CFU mL -1 of the *E. coli* and *S. aureus* strains was added to each concentration. The tubes were incubated at 35º for 24h. Sterility and growth controls were performed for the assay. After the incubation period, the MIC of the EO was verified, being defined as the lowest concentration that visibly inhibited bacterial growth (absence of visible cloudiness). Tests performed in triplicate.

For the Minimum Bactericidal Concentration (MBC) assay, an aliquot of 100 μL of the dilutions from MH broth that visibly inhibited microbial growth was used. The aliquots were inoculated in Mueller Hinton Agar (MHA) with subsequent incubation at 35°C for 24h. The MBC was determined as the lowest dose that visually in the MIC assay showed growth inhibition and that in the culture in AMH also did not present bacterial growth.

*2.9. Antioxidant activity*

The determination of antioxidant activity was performed by the ABTS method [2,2-azinobis-(3- ethylbenzothiazolin-6-sulfonic)], according to the methodology suggested by [23]. The ABTS•+ radical was prepared by the 5.0 mL reaction of a 3840 μg mL-1 solution of ABTS with 88 μL of the 37,840 μg mL-1 potassium persulfate solution, the mixture was left in a dark environment for 16 hours. After radical formation, the mixture was diluted in ethanol until absorbance of 0.7±0.01 to 734 nm was obtained.

From the concentrations of The EOs (5 to 150 μg mL-1) the reaction mixture with the radical cation ABTS was prepared. In a dark environment, an aliquot of 30 μL of each concentration of The EO was transferred in test tubes containing 3.0 mL of the radical Cation ABTS and homogenized in a tube agitator and after 6 minutes the absorbance of the reaction mixture was performed in spectrophotometer in length of 734 nm.

The capture of the free radical was expressed as a percentage of inhibition (%I) of the radical cation ABTS according to Equation 1 [24], where ABSABTS represents the absorbance of the ABTS radical solution and ABSAM represents the absorbance of the sample

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| --- | --- |
|  | Eq. 01 |

From the obtained data, the efficient concentrations IC50 and IC90 were calculated, defined as the concentration of the sample necessary to kidnap 50% and 90% of the ABTS radicals. The EO is considered active when it presents IC50 < 500 μg mL-1 [25].

*2.10. Collection of eggs of Aedes aegypti*

The eggs were collected at the Federal University of Maranhão, Bacanga Campus in São Luís/ MA, through traps called ovitrampas. These consist of brown buckets (500 mL), polyethylene, with 1 mL of beer yeast and 300 mL of running water and inserted two reeds of Eucatex for mosquito eggposition. The traps were inspected weekly for the replacement of the vanes and egg collection and sent to the Laboratory of Research and Application of Essential Oils (LOEPAV-UFMA) of the Technological Pavilion of the Federal University of Maranhão - UFMA. Initially, the eggs of *Aedes aegypti* were placed to hatch at room temperature in a circular glass aquarium containing mineral water. The identification of the species followed the methodology proposed by [26]. The larvae obtained were fed cat feed according to [27] until they reached the third and fourth stage, the age at which the experiments were carried out.

*4.11. Larvicidal activity*

The tests for larvicidal activity were performed according to the adapted methodology proposed by [28]. Initially, a 100 mg L-1 mother solution of each of the EOs was prepared and diluted in 2% DMSO solution. Five dilutions were prepared at concentrations 10, 20, 50, 70 and 90 mg L-1. 10 larvae were added at each concentration in the proportion of 1 mL/larva.

All tests were performed in triplicates and as a negative control a solution formed of DMSO 2%, and as a positive control, a solution of temephos (O,O',O'- tetramethyl O,O'-tiodi-p-phenylene bis (phosphothiotilate) at 100 ppm, equivalent to concentration used by the National Health Foundation (FUNASA) for the larvicide control of the vector, in addition to Novaluron (±-1-[3-chloro-4-(1-1-3-trifluro-2-trifluoromethoxyethoxy) phenyl-3-(2,6-diflurobenzoyl) urea at 0.02 mg L-1, dose adopted by the urea adopted by the ministry of health, which indicates by who in the range of 0.01 to 0.05mg L-1.

After 24 h, the count of live and dead were performed, and the larvae that did not react to touch were considered dead after 24 hours of the beginning of the experiment. To quantify the efficiency of the EO, the statistical test [21] was applied.

*4.12. Obtaining and cultivation of Biomphalaria glabrata adult snails*

Samples of adult snails of the species *Biomphalaria glabrata* were captured in the rainy season, in areas free of low sanitation in the neighborhood Sá Viana, São Luís-MA. No permission was required for the collection of the animals. The collection technique was performed according to a proposal from [29], performing a scan with a shell in the submerged areas and the captured snails were collected in a glass container with lid, with water from the breeding site itself. Their search was carried out at various points in each breeding site, and then sent to the molluscum of the Laboratory of Research and Application of Essential Oils (LOEPAV/UFMA).

The snails were kept in the laboratory for 30 days and analyzed every 07 days to confirm the absence of Infection by *Schistossoma mansoni*. For this, 05 snails were placed in transparent glass containers with 25 mL of dechlorinated water, that is, 5 mL/snail, exposed to light (60 W lamps) for one hour with a distance of 30 cm to stimulate the release of the fences and taken to be analyzed, through visualization with the aid of a stereoscopic magnifying glass (8x), those that were parasitized (positive) were labeled and separated for future individual analysis and those who showed no signs of trematoid infection in the period of 30 days were selected for the molluscicide activity test.

*4.13. Evaluation of molluscicidal activity against Biomphalaria glabrata*

For the evaluation of molluscicide activity, the technique recommended by the [30] was used, where two tests were performed in triplicate. In the first, called pilot test, a solution of the oil under study was prepared in a volume of 500 mL at the concentration of 100 mg L-1 and 0.15 mL of Tween 80 (active tense), where 10 adult snails were placed, negative for *Schistossoma mansoni*, obtaining in the end a proportion of 50 mL/snail and feeding them with hydroponic lettuce.

They were exposed in the solution for 24 h, at room temperature, removed from the solution, washed twice with dechlorinated water, placed in a glass container containing 500 mL of dechlorinated water, feeding them with hydroponic lettuce and observed to every 24 hours for 4 days to assess mortality.

In the second test, called lethal concentration (LC50), solutions of each oil were prepared in a volume of 500 mL at concentrations 75, 50, 20, 10 and 5 mg L-1 and 0.15 mL of Tween 80 (surfactant), using the same methodology of the pilot test. For the negative control, two tests were also used, in the first we placed 500 mL of dechlorinated water and 10 snails in a glass container and in the second 10 snails immersed in a solution with 0.15 mL of Tween 80 in 500 mL of distilled water, feeding both with hydroponic lettuce and the analysis also performed in the previous tests. After the experiment, the remaining live snails were disinfected and killed using 1:10 solution of sodium hypochlorite, and were later discarded. Niclosamide was used as a positive control.

The lethal concentration LC90 of the bioassay was determined by linear regression, obtaining the concentration versus mortality ratio of mollusks. Mortality rates were obtained by averaging dead individuals as a function of the logarithm of the tested dose. The statistical analysis of the data for the LC50 was performed according to the [21].

**3. Results**

*3.1. Physicochemical parameters*

The physicochemical parameters are of great interest not only to determine the quality of a given product, but also to verify its purity. Foram obtidos os seguintes parametros: Refractive index 1,5300 (nD 25°); Density 0,9980 (g mL-1); Color Yellow; Yield 2,23 %.

*3.2. Total phenolics and antioxidant activity*

The total phenolic content of the sample analyzed was 220,12 ± 5,22 mg EAT g-1, which can infer a significant amount of the compound. The EO showed antioxidant activity through the ABTS method used with an IC50 of 100.36 ug mL-1 (R2=0,9970), and the percentage of maximum inhibition of radicals in 97.33%.

*3.3. Chemical constituents*

Figure 1 corresponds to the chromatogram of annatto, in which the presence of 20 signals between 3 and 21 minutes of chromatographic running is observed. Table 1 shows the components found in the annatto EO with their respective retention times, consisting of 20 identified components, which can confirm the total of 100% of the composition. The majority constituents were 1R-α-pinene (26.50%), β-bisabolene (19.71%), caryophyllene (10.42%) and pinene (9.23%), belonging to the classes of terpenes, monoterpenes and sesquiterpenes.

3.4. *Toxicity*

Table 2 presents the lethality assay against *Artemia salina* and its subsequent classification according to the criterion [31]. All larvae presented mortality against positive control. No larvae presented mortality due to submission to negative and white control.

*3.5. Bactericidal activity*

Table 3 contains the results obtained in antimicrobial assays and the statistical values obtained by the ANOVA standard.

*3.6. Larvicidal activity in front of Aedes aegypti*

Larvae mortality was observed at concentrations of 100; 70; 50; 30; 20 and 10 mg L-1 in 100; 100; 70; 70; 50 and 30%, respectively, during the 24-hour exposure. Table 4 shows LC50 and LC90 referring to the action of *B. orellana* L. EO in front of the larval form of *Aedes aegypti*. All larvae presented mortality against positive control. No larvae presented mortality due to submission to negative and white control.

*3.7. Mollusicidal activity against Biomphalaria glabrata*

Table 5 presents the results obtained in the tests for molluscicide activity and LC50 and LC90 can be observed through Table 5. The LC50 observed in 24 hours were lower than those observed in 72 hours. All snails presented mortality in relation to positive control. No snail presented mortality due to submission to negative and white control.

When we compare the concentration (mg L-1) with time (h) we observe that with increasing concentration and time mortality also increases, that is, thus evidencing that time is an indicator of mortality. According to Table 5 as time passes mortality increases and when time reaches 72 hours, mortality is 100% this shows that the EO of *B. orellana* has gradual action when applied as molluscicide.

**4. Discussion**

Using the hydrodistillation process to obtain the EO of *B. orellana* L., a yield of 2.23% can be observed. By comparing this yield to the results obtained by who extracted the EO from the dry annatto leaves and obtained a yield of 0.21%, one can infer the importance of using the EO of this study, since a value was found well above the yield maximum obtained by the author.

According to [33] such differences may be related to plant age and environment, and genetic characteristics. According to [34], one of the most important factors is the collection of plant material, since the number of constituents and their nature may vary according to the time of year. The development and age of the plant, as well as the different parts of the plants can influence the relative quantities of the components of the mixture, as well as the total proportion of the metabolites produced.

Studies aimed at evaluating the physicochemical parameters of *B. orellana* L. EO were scarce, making it difficult to analyze other factors. However, they are in accordance with the criteria established by the [35], and it is important to emphasize the yield of 2.23% of the EO that was observed in results higher than the literature, encouraging its production.

Using the same methodology [36], they quantified the total phenolic content of the leaf extract of *B. orellana*, evaluating the effect of the extraction time and the solvent/leaf ratio, obtained a slightly similar result, with a total maximum phenol content of 144.77 ± 9.66 mg EAT g-1. [37] reported total phenol concentration in ex-vitro cultivated leaves of 4,405 ± 0.391 μg mL-1, while the concentration of phenols in branches was 2,622 ± 0.2426 μg mL-1. [38] found total phenolic contents of five genotypes analyzed with mean values of 776.02 to 1,498.48 mg GAE 100 g-1 sample (dry weight) and 297.08 to 450.97 mg GAE 100 g-1 for hydroethanolic and ethanol extracts.

The concentration of phenolic substances present in natural products depends on factors such as season and environmental factors, as well as soil type and climate, genetic factors and processing methods [39]. The total phenolic content can be used as a powerful indicator of antioxidant capacity, which can be used as a preliminary screening for any product [39].

Phenolic compounds are indicated as considerable bioactive compounds, associated with several favorable health effects, among other functions, are mainly related to antioxidant activity in plants [40].

There are different methods to determine antioxidant activity, which depends on different free radical generators acting through different mechanisms [41].

The result presented is satisfactory according to the criteria of [42], classifying the IC50 of the EO as of strong inhibition. Few studies have examined the antioxidant activity of extracts and scarcity of the activity of annatto EOs, although [43] found that *B. orellana* leaf extract showed an activity of elimination of free radicals as well as [44]. Lower results are observed for other parts of the nnatto, since when analyzing the activities of elimination of DPPH radical from *B. orellana* seed powder at different concentrations (1.0-10 mg mL-1), [45] observed a variation of 85.8 94.7%, and the concentration at 5000 μg mL-1 showed the highest capacity.

The study of antioxidant potential is also of great ecological and physiological importance, because, in the face of a condition of oxidative stress, organisms such as algae, animals and terrestrial plants use these antioxidants as one of the main fronts [46].

There are numerous studies that show the existence of different chemical compounds among essential oils of different varieties and species. The EO obtained from the seeds of *B. orellana* L., have been the subject of several studies, however few studies report on the EO of the leaves of this plant.

The results of research on the chemical constitution of the EO of *B. orellana* L. leaves were scarce and different from those found in this study, however some similarities are noted regarding the presence of some components.

In a similar study conducted by [32] on the EO of annatto leaves, 28 substances were found, which reached 75% of the total constitution, of which α-humulene (43.01%), E-nerolidol (14.40%) and spathulenol (7.57%) were the majority constituents, belonging to the class of sesquiterpenes [47] identified 35 chemical components in the EO of annatto seeds, including farnesyl acetate (11.6%), occidentalol acetate (9.7%), spathulenol (9.6%) and ishwarano (9.1%). It also presented the presence of α-pinene (2.8%), β-pinene (2.5%) and β-bisabolene (1%), elements also found in this study, however in higher percentages

From the compositions of the annatto EO mentioned, it is possible to notice that 1R-α-pinene is not reported as a major constituent, despite the fact that in this research a representative relative abundance of this compound was obtained. The presence of 73 chemical components in the EO of *B. orellana* L. by [12] was detected, of which it was possible to identify 80%. Among them, the presence of α-pinene (3.3%) and β-pinene (2.3%), in much lower concentrations than those found in this study.

[11] established a criterion that determines the toxicity of natural products seeking a specific application of the agent in the target organism, and oils with high toxicity are not recommended for biological applications. Thus, the Lethal Concentration 50% (LC50) refers to the point where the number of surviving animals is equal to the number of dead animals. Since then, it is considered that when values of LC50 ≤ 80 mg are verified. L-1 indicates that the product is highly toxic when LC50 values range between 80 mg. L-1 and 250 mg L-1 denotes moderate toxicity and when values above 250 mg are found. L-1, these are considered nontoxic.

Based on this relationship, according to Table 2, it was possible to observe that annatto essential oil did not present toxicity because it exhibited LC50 a value higher than the reference to classify it as nontoxic, so this EO has its acceptable and encouraged application potential.

Since the effects caused by a compound in laboratory animals allow them to be applicable to humans, it makes toxicity tests such as the bioassay with *Artemia salina* valid and of the utmost importance. This bioassay is simple to handle, because it does not require special equipment or aseptic methods, besides being fast, low cost, favors its use in the routine of the laboratory itself [48].

In a study developed by [49], when using extracts from *B. orellana* leaves, evidenced a non-toxic effect against *Artemia salina*, these data corroborate the results of this study. However, [50] when testing the toxicity of the oily fraction of annatto, obtained the LD50 value of 285.41 ± 81 μg mL-1, classifying it as highly toxic and unfeasible to be used for biological tests.

The results presented are satisfactory when compared to those of [51] who evaluated the larvicidal activity of aqueous plant extracts in larvae of *Aedes aegypti* and observed that the aqueous extract of *B. orellana* had an effect moderate as larvicidal, with 15% preparation, after 72 hours of exposure to treatment, since in this study the concentration of 10 mg L-1 of The EO presented lethality of 30% against the larvae.

Studies using *B. orellana* as larvicidal are relatively new and scarce for *Aedes* larvae. However, seed EO, reported by Jondilko [52], has repellent and larvicide activities against female adults *Anopheles gambiae* and larvae of the third instar revealing that Oes are dosed between 0.2-0.7 mg mL-1 for 2-5 hours. Seed extract revealed mortality and repellency of larvae of *Rhynchophorus palmarum* L., Pijuayo *Bactris gasipaes*, Kunth Plague insect in the Peruvian Amazon [53].

The advantage in the use of natural products such as *B. orellana* EO as sources of a complexity of molecules is that when combined they exhibit various bioactivities, and the toxicity levels of individual chemically pure compounds are very high compared to and at the risk of generating resistance [54].

According to criteria established by [55], the inhibition halos formed allow classifying all bacterial strains as sensitive against The EO of *B. orellana* L. Annatto EO was more efficient in inhibiting bacterial growth of *S. aureus* (20mm) when compared to *E. coli* that presented a halo of 13 mm. This result is satisfactory since previous studies have reported this action in plant extracts with values lower than that of this study.

[56] when evaluating the microbial inhibition activity of methanol extracts obtained from seeds of different genotypes of *B. orellana* L. (annatto) at different stages of maturation, found that all extracts showed activity of sensitivity on the bacterium *S. aureus*, presenting halo values with diameters ranging between 10-18 mm, while for *E. coli* these did not present inhibition halo. On the other hand, [57], in a similar study, found that the inhibition of growth of *E. coli* and *S. aureus* was significantly high for annatto leaf extract at a concentration above 1500 μg mL-1.

However, [58] when analyzing the antimicrobial action of hydroalcoholic extract of annatto in the face of 10 bacterial strains by the diffusion disc method, verified sensitivity of *S. aureus*, with a halo of 22 mm, a value higher than that found in this research.

Some authors have analyzed the bactericidal activity of the plant *B. orellana* L. (annatto) for the bacteria *S. aureus* and *E. coli*, but through other techniques for assessing antimicrobial sensitivity. [59] and [60] observed effective antibacterial activity mainly for Gram-positive bacteria such as *S. aureus*, E. faecalis and L. monocytogenes, while gram-negative bacterium *E. coli* presented the lowest Sensitivity. Nevertheless, [61] demonstrated that the ethanol extract of annatto leaves obtained a higher inhibition zone against the bacterial strain of *E. coli*, presenting 22.5 mm in diameter.

According to [62], the classification of antimicrobial activity for plant specimens, according to the results of the MIC, is considered strongly inhibited: MIC up to 500 μg/mL; moderate inhibition: MIC between 600 and 1000 μg mL-1; and weak inhibition: MIC above 1000 μg mL-1.

The MIC value of The EO of *B. orellana* L. against the strains of *E. coli* and *S. aureus* were 400 μg mL-1 and 250 μg mL-1, respectively, according to Table 3. From the results obtained, it is stated that the annatto EO presented strong inhibition potential compared to all bacteria tested in this study. However, in the analysis by [63], the authors reported weak inhibition of annatto leaf extract against *S. aureus*, which exhibited AM of 4000 μg mL-1.

The assay for MBC showed better results for EO against *S. aureus*, observing bactericidal action from 500 μg mL-1, while action was observed from 700 μg mL-1 to *E. coli*. [64], observed similar results in relation to bactericidal action against *S. aureus*, with MBC of 625 μg mL-1. In a study conducted by [65], it indicated that the bactericidal effect of *B. orellana* hydroalcohol extract was achieved at higher concentrations (64 mg mL-1). A similar result was observed by [63], who presented bactericidal action from 16 mg mL-1 to *S. aureus* as well. Regarding this atvidity on *E. coli*, the studies indicated a slight bactericidal or nonexistent action. Antimicrobial substances present in EO may have influenced bactericidal activity in susceptible bacteria tested.

According to the [30], a plant to present molluscicide potential should present a LC90 below 100 mg L-1, thus classifying the OE used as active against the tested snails.

Table 5 shows that mortality is directly linked to increased EO concentration of *B. orellana*, as concentration increases mortality also increases. Studies in the literature on molluscicide activity in the *Biomphalaria glabrata* front of *B. orellana* EO are still scarce and little publicized.

[66] evaluated the molluscicide activity performed with the EO of the barks of *Citrus Limon* L. against *Biomphalaria glabrata*, found a LC50 13.18 mg L-1, a value lower than that found in this study. [67] verified the molluscicide activity performed with the EO of *Syzygium cumini* (L.) Skeels leaves identified α-pinene (31.85%), (Z) -β-ocimene (28.98%) and € -β-ocimene (11.71%) as main compounds and a LC50 90 mg L-1 compared to *Biomphalaria glabrata*, a value higher than that found in this study.

[68] evaluated the molluscicide potential obtained from the EO of the leaves of *Cinnamomum zeylanicum* Breyn, where the LC50 was 18.62 mg L-1, being within the established by the WHO (1983), in which it indicates that the plant should only be considered molluscicide when the concentrations equal to or less than 20 mg L-1 for extracts and 100 mg L-1 for the raw vegetable and may undergo field trials.

[69] under study with EO of *Xylopia langsdorffiana* A. St. Hil leaves. Tul et. (Annonaceae) found significant molluscicide activity against *Biomphalaria glabrata* (LC90= 5.6 mg L-1) identifying as major constituents monoterpene (α-pinene) and sesquiterpenes (germacreno D and caryophyllene), in addition to β-pinene and limonene (monoterpenes) in lower concentration. [70] investigated the composition and molluscicide activity of *Cymbopogon winterianus* EO cultivated in northern Brazil that presented LC90 97.0 mg L-1, higher than that obtained in this study.

**5. Conclusions**

In view of the results presented, it is concluded that the EO of the leaves of *B. orellana* L. presents antimicrobial activity on the strains of the bacteria Staphylococcus aureus and Escherichia coli. It demonstrated satisfactory lethal concentration against the larvae of *Aedes aegypti* and *Biomphalaria glabrata*. It was considered nontoxic, which emphasizes the importance of pointing out this product as a therapeutic alternative, encouraging its application potential.