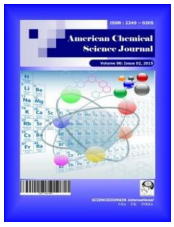

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Phytochemical Study, Microbiological Activity and Cytotoxicity of Leaves and Stem from Plant Species *Mikania lindleyana* DC

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Authors' contributions

This work was carried out in collaboration between all authors. Authors WAC, DHSS and SSMSA planned all experiments. Authors WAC, DHSS and RSR supported the chemical study and cytotoxicity of leaves. Authors WAC, DHSS and ELC supported the study of the microbiological activity. Authors RSR and SSMSA wrote the first draft of the manuscript. Author LRT performed the correction of English throughout manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of this research was to study phytochemical, antimicrobial activity and cytotoxicity of crude ethanol extract of *Mikania lindleyana* leaves and stems.
Study Design: Samples of plant species *M. lindleyana* were collected in the city of Belém, Pará State, Brazil. The leaves and stems of this species were analyzed and the plant was identified by

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comparing with the voucher specimen at the Herbarium of the Federal University of Amapá, in the city of Macapá. Experimental tests were performed at the Laboratory of Pharmacognosy and Phytochemical of the Pharmacy Undergraduate Course at the Federal University of Amapá.
Place and Duration of Study: The samples collection occurred in the city of Belém-Pará-Brazil in December, 2012. The study was conducted between January and September, 2013 at the Federal University of Amapá.
Methodology: The phytochemical screening was performed according to the method described by Macêdo and by the Brazilian Pharmacopoeia. The bioassay cytotoxicity on *Artemia salina* was performed using the ethanol extracts of the *Mikania lindleyana*, where calculations were made for the CL_{50} employing BioEstat ® and PROBIT tool. The microbiological test was performed by the tube microdilution method in *Escherichia coli* and *Staphylococcus aureus*.
Results: The Screening Phytochemical analysis of the leaf and stem ethanol extract from the *Mikania lindleyana* DC indicated the presence of tannins, saponins and the extracts showed no antimicrobial activity on the bacteria tested. The cytotoxicity of the extracts might be related to the alkaloids and saponins present in its constitution.
Conclusion: The study helped the community, suggesting that despite its popular use is not recommended as antimicrobial agent, and contributed to the scientific community, opening a new front of research.

Keywords: Medicinal plants; secondary metabolites; biological activity; Mikania lindleyana.

1. INTRODUCTION

Medicinal plants are widely used as curative purpose, and they are essential to health care [1,2], whose knowledge is based on traditional use [1,3]. Nowadays, the employment of plants as medicinal resource has increased, due to the high cost of drugs and medications and the difficulties for the people to access health care [1,4].

The Asteraceae is one of the largest plant families representing 10% of the world's flora [5]. According to Prudêncio [6], it comprises 1,600 to 1,700 genera and 24,000 to 30,000 species. In Brazil, this flowering plant has about 250 genera and 2,000 species. The genres are usually small, species can be presented as shrub, herbaceous, tree, vines and herbs [6]. The Asteraceae family is of much interest to health, since there is scientific evidence regarding drug stock of plants of the family [7]. Worldwide, it is found in various locations, such as USA, Brazil, the Mediterranean, Asia and Australia [7].

The *Mikania* genus has around 450 species, and they are distributed over tropical areas in Africa, Asia and America. In Brazil, there is a description of about 200 species, which concentrates mainly on the north and southeast region [7]. Many species of this genus are of interest, used as medicinal plants, such as *Mikania lindleyana*.

The *Mikania lindleyana* DC, is popularly known in Brazil as sucurijuzinho, sucuriju among other names. This plant species only blooms underdirect sunlight conditions. It is commonly used as popular medicine in the Amazon region, and has the following applications: anti-inflammatory, analgesic, diuretic and antihypertensive [7-9]. Many people use this plant to prepare tea with fresh leaves for therapeutic purposes, without knowing the quality of the plant material, which becomes relevant to study this species [10].

This medicinal plant is a climbing shrub, perennial, woody and without tendrils (claws to hold) with voluble stem, fluted cylindrical, green and twiggy, belongs to the Asteraceae family and gender *Mikania*, originally from South America and Brazil. Presents opposite leaves of bright green color, petiolate, cordate, smooth consistency, almost leathery and triangular, entire board, with three ribs on the base.

According to Carvalho [11], phytochemical is the science that studies the chemistry of the plants. For Silva [9] these chemicals come from their own metabolism, which is divided into primary and secondary. Primary metabolites are essential for the growth and development of all vegetables, and are classified as carbohydrates, lipids and protein. Secondary metabolism products are restricted to a particular taxon, and may occur as alkaloids, saponins, tannins, anthocyanin glycosides and several others. They comprise a rich and diverse class of compounds intended to give properties and functional activities of a given plant. However, the quality of the raw plant material by itself does not guarantee the effectiveness and safety of the

final product. The phytochemical analyses, is used to qualitative testing for metabolites classes, and the physicochemical analysis associated with the pharmacological, partly ensures the effectiveness and quality of plant material [12]. Santana [13] defines the secondary metabolic products as active ingredients, whose function in plants can be: defense against pathogens, protection against ultraviolet rays, and attraction of pollinators. In humans, the active principles have functions to cause reactions in the organism, which may result in the recovery of health [14]. Among the many pharmacological reactions beneficial to the human being that the active ingredients cause, the antibacterial activity can be emphasized.

The first antimicrobial activity of plant extracts study of is dated of 1881 [13]. Natural resistance developed by bacteria to antimicrobials is troublesome [15,16]; and in recent years, the interest and search for antimicrobial herbal substances hasincreased [9]. Considering that the Brazilian Amazon has a great variety of medicinal plants, research efforts are done looking for species that have the presence of secondary metabolites with antimicrobial activity [17].

Nunes [18] points out that, in the sphere of the study of medicinal plants, it is necessary to perform cytotoxicity studies since the use of medicinal plants can trigger in humans adverse reactions by their own chemical constituents [19]. A cytotoxicity evaluation model that is emerging among researchers is the bioassay on *Artemia salina*. This method is widely used, due to its simplicity, speed and low cost, which favors its use in several studies of plant species [20].

2. MATERIALS AND METHODS

This study involved experimental, exploratory, qualitative and quantitative aspects. The pharmacognostic analyzes, the phytochemical screening and forward cytotoxicity activity on *Artemia salina* were held at the Pharmacognosy and Phytochemistry Laboratory of the Federal University of Amapá (UNIFAP). The microbiological assays were performed in the private clinical laboratory Biodiagnostics in the city of Macapá.

2.1 Obtaining Plant Material

Plant species of *M. lindleyana* were collected in the city of Belém, State of Pará, Brazil. The analyzed material included the leaves and stems of this species and the plant was identified by comparing with the specimens at the Herbarium of the Federal University of Amapá, in the city of Macapá, Amapá, Brazil.

2.2 Obtaining Extracts Crude

The leaves and stems of *M. lindleyana* were initially mechanically chopped, separately. Portions of 250 grams were subjected to a process of hot ethanol extraction, employing a reflux at 45℃ for 45 minutes, using 700 mL for each extraction, and this process was performed in triplicate. Then, the materials were filtered and the ethanol extracts were concentrated on a rotary evaporator, under reduced pressure, thus obtaining, Crude Ethanol Extracts of the leaves and stems of the plant species *M. lindleyana* (EBEFM and EBMCM, respectively) [21]. The extracts were used for phytochemical screening, microbiological analysis and cytotoxicity.

2.3 Pharmacognostic Analysis

2.3.1 Determination of Ph

The pH determination was made according to Macêdo [22] and the rules established by the Adolfo Lutz Institute [23]. Ten grams of plant material were weighed in a beaker and diluted with 100 mL of distilled water. The contents were stirred until the particles were uniformly suspended. The pH was determined using a calibrated pH meter.

2.3.2 Determination of lipids

Three grams of plant material were weighed on a filter paper and tied with woolen yarn. The tied filter paper was transferred to the Soxhlet extractor type device, which was connected to a 250 mL Erlenmeyer flask and heated in a heating mantle to 45℃, and he was seated on a refrigerator balls. Hexane (150 mL) was added to the heating flask. After removing the solvent, the flask was placed in an oven at 105℃ and maintained for one hour. After cooling in a dessicator for 30 minutes the Erlenmeyer was weighed and this process was repeated until obtaining a constant weight [22,23].

The calculation of percentage of lipids was done using the following equation:

$$\frac{100 \times \mathrm{N}}{\mathrm{P}} = \% \text{ lipids, m/m}$$

Where:

N = weight of lipids in grams
P = weight of the sample in grams

2.3.3 Determination of the residue by incineration - total ash

The total ash includes physiological ashes and non-physiological ashes. Three grams of powdered plant material were weighed and transferred to a porcelain crucible. The sample was evenly distributed in the crucible and incinerated at a temperature gradually increased from 200℃ to 600℃±25℃, until the total elimination of coal. The t ash was cooled inside a dessicator, and weighed [21-23].

The total ash percentage was calculated by the equation:

$$\frac{100 \times N}{P} = \% \text{ ASH, m/m}$$

Where:

N = weight of ash in grams
P = weight of the sample in grams

2.3.4 Moisture determination - gravimetric method

Petri dishes (6 units) were washed, dried, weighed and left in a dessicator. Three samples of leaf powder and three stem powder samples, with about 2 grams each were distributed on each Petri dish. Then, they were dried in an oven at 105℃ for 2 hours, cooled inside a dessicator for 30 minutes and weighed. This process was repeated until obtaining a constant weight [21].

The equation used for the calculation of the moisture was as follows:

$$\frac{Pu - Ps}{Pa} \text{ X } 100$$

Where:

Pa = weight of the sample in grams.
Pu = weight of the petri dish containing the sample before drying.
Ps = weight of the petri dish containing the sample after drying.

2.4 Screening Phytochemical

The phytochemical analysis were performed according to standard methodologies coloration and precipitation described by Simões [12].

2.5 Biological Activity

2.5.1 Antimicrobial activity

To determine the possible antimicrobial activity, the microdilution tube method was used. Two solutions were prepared, using EBEFM and EBECM, each with 10 mg of the sample and 10 mL of saline solution. Then 1 mL of the concentrates without anticoagulant were placed in vacuum tubes, and dilutions 1:2, 1:4, 1:8 and 1:16 were made. Bacterial suspensions were prepared in glass tubes with 300,000 bacteria per mL (Mcfarland scale), for *Escherichia coli* and for *Staphylococcus aureus*. After that 10 µL of the suspension were inoculated, corresponding to 300 bacteria per inoculation, on the concentrated plant extracts, and incubated at 36℃. After 1 hour the strains of *Escherichia coli* were inoculated on Eosin Methylene Blue (BEM) and the *Staphylococcus aureus* on blood agar; they were incubated again and for 3 hours at 36℃ [24].

2.5.2 Cytotoxicity activity of *Artemia salina* L.

2.5.2.1 Preparation of artificial marine solution

The saline solution was prepared with 34.2 g of sodium chloride; 1.425 g of magnesium sulfate; 4.75 g sodium bicarbonate and 951 mL of distilled water. After homogenized, the pH was adjusted to 9.0, using a solution of 2 mol/L of sodium hydroxide.

2.5.2.2 Obtaining A. salina metanauplios

To obtain the metanauplios, *A. salina* cysts were incubated in artificial marine solution (pH 9.0 and 28ºC) under artificial lighting (40W bulb light for 24 hours). After hatching, the metanauplios migrated through a perforated plate to another compartment with free incidence of light, due to its phototropism, thus separating the larvae of cysts which have not hatched. Later, the metanauplios were separated into petri dish and allowed to stay under artificial light for 24 hours to obtain the nauplio.

2.5.2.3 Sample preparation and bioassay

The bioassay employing *A. salina* was based on the technique described by Nascimento [25]. It was used 10 mg of EBEFM and EBECM, to which was added 1 mL of Tween 80 to 5% to help solubilize it. The solution was homogenized, and the volume was completed to 5 mL with artificial sea solution at pH = 9.0. From this

solution, it was withdrawn aliquots of 2500, 1875, 1250, 625, 250 and 125 µL and transferred separately to 5 mL vials. The volumes were completed with the same solvent, to give concentrations of 1000, 750, 500, 250, 100 and 50 µg/mL of each EBEFM and EBECM extracts.

The nauplio were divided into seven groups, each containing ten individuals. The first group received the control solution (marine solution) and the six *M. lindleyana* solution of extract in different concentrations. The samples were submitted to artificial light for 24 hours; after this period it was recorded living and dead larvae. Dead larvae were considered those that showed no active movement in about twenty seconds of observation. The experiment was performed in triplicate for each extract.

2.5.2.4 Statistical analysis

To obtain the total ash values and moisture content, Microsoft Excel 2007 was used. The calculations to determine the mortality rate of *Artemia salina* versus concentration of EBEFM and EBECM were executed using BioEstat® 5.0 program by PROBIT.

3. RESULTS AND DISCUSSION

According to the results of the pharmacognostic analyzes (Table 1), the pH of the leaves and stems suspensions were 5.56 and 5.46 respectively, that may characterize the presence of acidic substances This is probably related to the presence of saponins and tannins, because they tend to lower the pH, due to the release of H^+ ions in the reaction medium [12]. The result is similar to that reported by Owiti [10], which the value found for the sucuriju leaves was 5.0.

The values obtained for total lipids were 3.15% and 0.38% for leaves and stem respectively, but the residues obtained in the extraction were not only composed by lipids, but for all compounds that can be extracted by the solvent. Therefore, it is necessary a more rigorous analysis for the identification and qualitative determination for the adequate result quantification [12].

For moisture, the recommended maximum limit for herbs is 14% [12]. It was found that the powder of leaves and stems of *Mikania lindleyana* presented moisture content of 7.46±0.09 and 7.77±0.06 in percentage, which allows to predict that the plant material is protected from attacks by microorganisms and enzymatic actions that may modify its natural properties.

The values in percentage of total ash for leaves and stem of *Mikania lindleyana* were 17.29±0.99 and 1.41±0.91, respectively, compared to 8-14% limit established by the Brazilian Pharmacopoeia [21]. It was observed that the value for the leaves are above recommended levels, suggesting a high concentration of inorganic material [26]. Owiti [10] reported the amount of total ash for sucuriju leaves of 17.067±0.5%, which is similar to that found in this study.

Table 1. Analysis pharmacognostic leaves and stem of the plant species *Mikania lindleyana* DC

Physical chemistry analysis	Leaves	Stem
pH	5.56[a]	5.46[a]
Lipids (% m/m)	3.15[a]	0.38[a]
Total ash (% m/m)	17.29±0.99	1.41±0.91
Humidity (% m/m)	7.46±0.09	7.77±0.06

[a] - Standard deviation null

The phytochemical research provided the detection of secondary metabolites, which are: alkaloids, glycosides anthocyanins, tannins and saponins (Table 2). These results are similar to those reported by Mendes [27] who studied the alcoholic extract from sucuriju leaves and found alkaloids, tannins, steroids and triterpenoids. In the study by Silva [7] this author showed the presence of saponins in the leaves of this species.

Table 2. Screening phytochemical leaf and stem of the plant species *Mikania lindleyana* DC

Class metabolites	Leaf	Stem
Alkaloids	+	+
Anthraquinones	-	-
Steroids and triterpenoids	-	-
Anthocyanin glycosides	+	+
Saponins	+	+
Tannins	+	+

(+) Presence (-) Absence

The presence of these secondary metabolites groups have pharmacological interest and justifies the popular use of the plant as healing. The presence of tannins [28] helps to form a protective layer on injured epithelial tissues, allowing just below this layer, the occurrence of tissue repair process [29]. Anti-inflammatory and antinociceptive functions, through the alkaloids, may act biologically to interfere mediators involved in the inflammatory process [12]. Saponins are able to lower cholesterol levels, by increasing its excretion, which occurs due to its ability to bind to sterol; therefore, a hypolipidemic

agent [12]. The anthocyanin glycosides are antioxidants metabolites, slowing or preventing oxidative damage, by blocking the oxidation reactions [12].

For antimicrobial activity, the results demonstrated that the EBEFM and EBECM showed no activity over the strains of *E. coli* and *S. aureus*, considering that the concentration of 1000 µg/mL is toxic. Thus, it is irrelevant to further testing at higher concentrations. The results are shown in Tables 3 and 4.

The cytotoxicity bioassay on *Artemia salina*, provided the LC50 for EBEFM and EBECM equal to 619.6 and 637.0 µg/mL respectively, assigning cytotoxic activity to both extracts. Nascimento [25] considers that the quantity below 1000 µg/mL as cytotoxic, which can be related to the cytotoxic activity of alkaloids and saponins [30]. The concentration-response curves of the *Artemia salina* mortality after 24 hours submitted to EBEFM and EBECM extracts. are shown in Graphics 1 and 2, respectively.

Table 3. Growth of *E. coli* strains in EBEFM and EBECM concentrated and diluted *Mikania lindleyana*

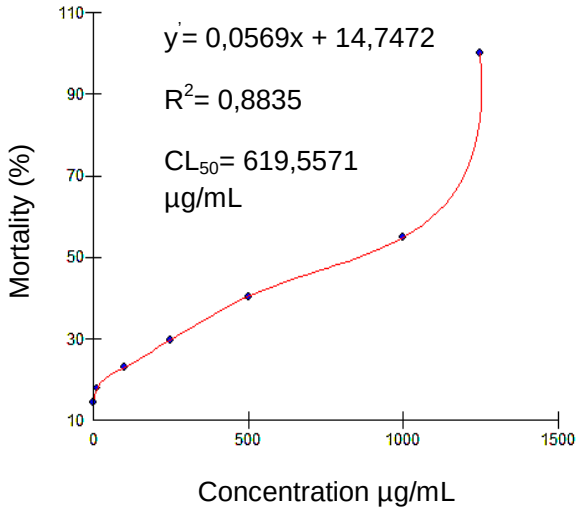
Sucuriju extract dilution	*E. coli* EBEFM 1 hour	*E. coli* EBECM 1 hour	*E. coli* EBEFM 2 hours	*E. coli* EBECM 2 hours	*E. coli* EBEFM 3 hours	*E. coli* EBECM 3 hours
1000 µg/mL	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	+
1:8	+	+	+	+	+	+
1:16	+	+	+	+	+	+

(+) Growth

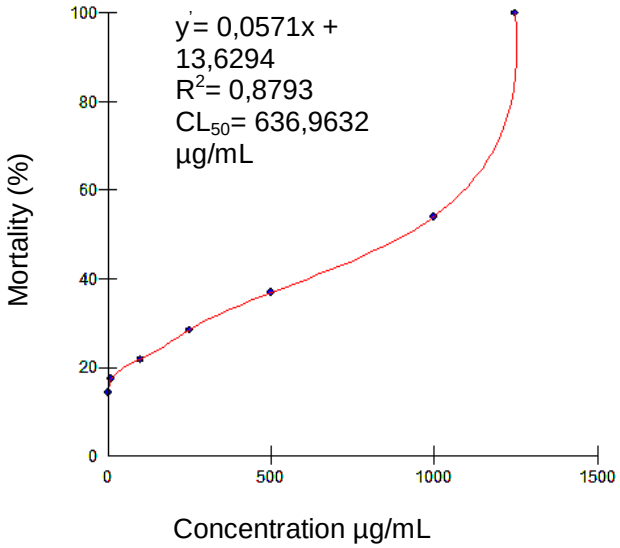
Table 4. Growth of *S. aureus* strains in EBEFM and EBECM concentrated and diluted *Mikania lindleyana*

Sucuriju extract dilution	*S. aureus* EBEFM 1 hour	*S. aureus* EBECM 1 hour	*S. aureus* EBEFM 2 hours	*S. aureus* EBECM 2 hours	*S. aureus* EBEFM 3 hours	*S. aureus* EBECM 3 hours
1000 µg/mL	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	+
1:8	+	+	+	+	+	+
1:16	+	+	+	+	+	+

(+) Growth



Graphic 1. Concentration-response curve of the *Artemia salina* mortality after 24 hours submitted to EBEFM extracts



Graphic 2. Concentration-response curve of the *Artemia salina* mortality after 24 hours submitted to EBECM extracts

4. CONCLUSION

Through pharmacognostic analysis, it was possible to obtain quality control parameters consistent with the Brazilian Pharmacopoeia. The pH of the leaves and stems of *Mikania lindleyana* showed acid characteristics, because of the potential ability of tannins and saponins to release ions H^+ in the aqueous medium. In the lipid analysis, residues obtained in the extraction were not formed only by lipids, but for all compounds under the conditions that could be extracted by the solvent. Thus, a more rigorous analysis for the qualitative detection and therefore the identification and quantification are necessary.

Moisture from the leaves and stem of the *Mikania lindleyana* were consistent with the values suggested by the Brazilian Pharmacopoeia, which allows to predict that the plant material is protected from attacks by microorganisms and enzyme action that may modify its natural properties. The value of total ash to leaves were high compared to the Brazilian Pharmacopoeia standards, which possibly corresponds to a high concentration of minerals on its constitution, which did not occur with the stem; this part of the plant showed little concentration of minerals.

The phytochemical screening justified the popular use of the studied species as healing, anti-inflammatory and analgesic, through the biological action of tannins and alkaloids, plus add other possible activities. Also it may act as lipid-lowering and antioxidant by the presence of saponins and glycosides anthocyanin, respectively, what makes this study relevant for society and collaborating to the chemosystematics of the species.

The EBEFM and EBECM did not have antimicrobial activity over *Escherichia coli* and *Staphylococcus aureus* which does not exclude the possibility of developing antimicrobial activity related to other bacterias.

The bioassay on *Artemia salina*, contributed to the population, warning about the possible toxic concentrations of the species under study, and contributed to the scientific community, opening a new front of research, whereas the saponins have cytotoxic action against anti-tumor cells and that many cytotoxic drugs of plant origin are used in cancer treatment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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