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Phytochemical Study and Evaluation of *Euphorbia Serpens* Plant Extracts Biological Activity

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Abstract
The chemical composition of the plants of the *Euphorbia serpens* Kunth and its antimicrobial properties can make a significant contribution to the improvement of the quality of life, scientifically arguing its use in the population as a way to combat microorganisms resistant to traditional drugs, to promote the rational and economical management of antibiotics. This research contributes to the phytochemical knowledge of the plant species *E.serpens*, providing information about the possible active components present in the aerial parts of the plant, thus generating a scientific impact. This study contributes, in the same way, to leave open an investigation on a possible formulation with the fractions and the active extract, since the expected results in this project are to determine if this species has a biological activity or not, thus determining the different potential uses of the same. It is becoming an innovative product at the pharmaceutical industry level that reduces pathologies related to microorganisms, which today are a public health problem in our country.

Keywords
Euphorbia serpens, leaf extracts, Photochemistry, biological activity

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1.0 INTRODUCTION
In the rural Indian population, the use of plants for therapeutic purposes is closely linked to cultural traditions. Currently, phytochemistry has awakened awareness of change in scientific and academic generations; many studies describe the medicinal importance, taxonomy and pharmaceutical uses of species of the genus Euphorbiaceae. The Euphorbiaceae family comprises about 8100 species; it is common in tropical countries and is among the most diverse families among the Euphorbiaceae[1]. The species of Euphorbiaceous (*Euphorbia serpens* Kunth) have great prominence in economic activity through human nutrition and medicine based on widespread knowledge. Although this family is widely distributed in India, some of its species have never been studied regarding its use, phenology, or chemical composition; in addition, morphologically, many medicinal species are not classified, which makes it a potential family in bioactive products that may involve more collections and indeed many uses could be confirmed. Many of these species are

currently objects of multidisciplinary studies involving additional research on their chemical characteristics, biological properties, ethnobotanical and taxonomic importance, contributing to a better understanding of this family. According to the phytochemical studies carried out by Salehi et al. (2019) [2], the Euphorbiaceae family is used for medicinal purposes, such as the *Euphorbia serpens* is characterized by having astringent characteristics. Given the background described, the information provided in this study fills a knowledge gap since, although the species is widely used, its chemical composition and biological activity are unknown. In traditional medicine, the use of species of the Euphorbiaceae family is varied and can be used in dermatology as a fungicide and antimicrobial to treat gastrointestinal diseases and bacterial infections such as gonorrhoea [3]. In the study by Dahiya et al. (2012) [4], it was proposed to relate the therapeutic properties of the extract with the biological activity against microbial agents, which is why different techniques were used for the phytochemical analysis of antimicrobial activity on various bacteria. In the present study, the compounds present in *Euphorbia serpens* following the studies of other researchers [5-6], giving the necessary guidelines for using this plant species that grows in India for its subsequent use in traditional medicine. The current study carries out the preliminary phytochemical analysis of the leaf extract of the plant species *Euphorbia serpens*. It evaluates its biological activity and toxicity to establish a potential use of this species.

2.0 MATERIALS AND METHODS

The taxonomic identification of the species *Euphorbia serpens* Kunth (Figure 1) was carried out on October 22, 2019, by a curator of the Herbarium of the Department of Botany Acharya Nagarjuna University, Guntur, India. One of the most widely dispersed species in the New World is *Euphorbia serpens*. While it is native to a portion of the flora region, most likely in the warmer, southern regions of its habitat, it is a weed that has likely spread to many other parts of the flora region, including South India. It's also readily available in India, where it was probably introduced. The stems are rooted at the nodes, rigidly prostrate the habit.[7]

Classification

Order: Malpighiales
Family: Euphorbiaceae
Genus: Euphorbia
Species: *Euphorbia serpens* Kunth

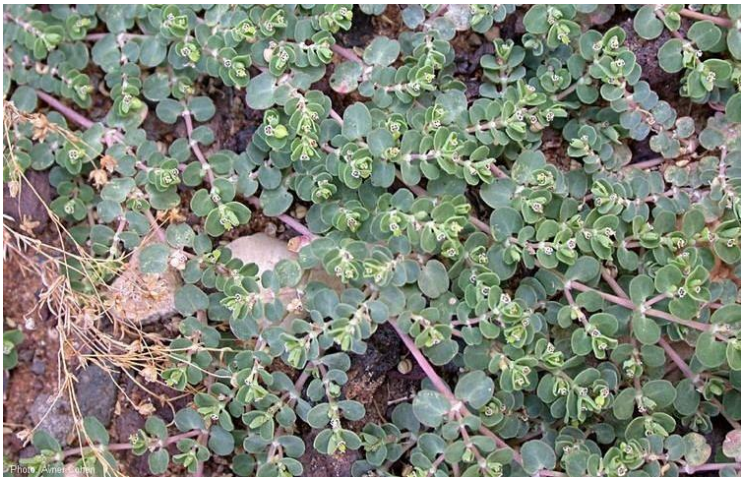

Figure 1. Plant *Euphorbia serpens [8]*

2.1 Sample collection: It is made up of the plant species *Euphorbia serpens* Kunth, which is located in the District of Guntur agricultural fields (Pedavadlapudi: 16.4115° N, 80.6110° E), Andhra Pradesh, India The leaves of the *Euphorbia serpens* Kunth species that were collected in the Pedavadlapudi agricultural fields. The leaves of *Euphorbia serpens* Kunth were selected and weighed and then dried in a forced-air oven at an average temperature of 50°C for approximately 24 hours and then ground and weighed again. These dried and ground leaves were stored in bags with a zipper closure and stored in a drying hood to prevent the passage of moisture.

2.2 Preparation of extracts: The aerial parts (leaves) of *E.serpens* dried at room temperature; (450 g) of dry material underwent cold maceration with 96% ethanol in a glass container, left to rest for five days in a cool, shaded place. Then, filtration was carried out to separate the extract from the solid residue, and finally, the obtained extract was concentrated in a Laborota 4000 rotary evaporator. The crude extract was weighed and placed inside a filter paper cartridge and introduced into the body of the Soxhlet

extractor. The assembly was carried out, and the extraction was left for three h with each of the solvents used. The fractionation was started with hexane (reagent grade: for analysis, brand: Merck), then with chloroform (AR grade: brand: Merck), continued with butanol (AR grade, brand: SD fine India) and finally, with 96% ethanol. The fractions obtained were concentrated in a rotary evaporator and left in an oven at 30°C to bring them to dryness. The extracts obtained were gathered to determine the phytochemical profile and evaluate the biological activity.

2.3. Determination of the phytochemical profile.

The crude extract was analyzed to determine the presence of groups of secondary metabolites according to the Sanabria-Galindo methodology (1983)[9]. This methodology uses various precipitation and staining reactions to reveal the presence of specific groups of secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, steroids etc.,

2.4 Biological studies

2.4.1 Toxicity test against *Artemia salina*

Artemia salina eggs were added to a 500 mL container with a solution composed of 3.8 g of sea salt per 100 mL of water at a temperature of 27 °C to allow hatching. The nauplii were left in the solution for 48 h, and after this time, they were exposed to the extract at different concentrations [11]. This assay was performed in multiwell culture plates with a capacity of 5 ml. A Stock solution of crude extract (1000 µg/mL) in ethanol was prepared, where it was evaluated at concentrations of 10, 50, 100, 200 and 500 µg/mL, which were prepared using solutions taking from each one a volume of 30, 150, 300, 600 and 1500 µL and water control was carried out for each well [11]. The crustaceans were collected with a glass Pasteur pipette and transferred into wells of the plate at different concentrations in quadruplicate, consecutively completing with a saline solution medium. The plates were incubated at room temperature, and after 24 hours, the number of dead larvae in each plate was determined. If there is no movement in the appendages for 10 seconds, the larva is considered dead [11].

After performing the count, the percentage of mortality is calculated using the probit statistical program, which consists of a regression that relates the dose to the response and calculates for each percentage of the population the lethal dose 50 (LD_{50}) that corresponds to the concentration that causes the death of 50% of the population [12].

2.4.2 Antimicrobial activity assays

Initially, gram staining was performed to visualize the yeast *Candida* sp microscopically, and subsequently, the different bioassays were performed. The antimicrobial activity was evaluated using the agar diffusion method. The crude extract and the different fractions were analyzed against microorganisms: *S. aureus* ATCC 25923, *E. coli* ATCC 25922 from reference strains. At the same time, *Klebsiella* sp and *Candida* sp were isolated from the certified strains from commercial suppliers. These microorganisms were selected due to their clinical importance since they are the cause of infectious diseases transmitted directly or indirectly to men, such as respiratory infections and gastroenteritis. Mueller Hinton (MH) agar was used for the bioassays, and a suspension of each microorganism was prepared in 0.085% saline solution according to the McFarland pattern that corresponds to a concentration of $1.5x10^8$ cells per millilitre, for which 4 to 5 colonies of each microorganism with a bacteriological loop and placed in 5 ml of saline solution.

The bacteria were seeded with the help of a sterile swab on Mueller Hinton (MH) agar, which was prepared, following the manufacturer's instructions, 34 g of agar for each medium in one litre of deionized water and after 15 minutes of rest, it was sterilized for 45 minutes, and the microorganism was allowed to fix for 15 minutes. In the laminar flow cabinet, 20 mL of agar were poured into each petri dish and allowed to solidify for 20 minutes [13].

2.4.2.1 Antimicrobial evaluation

The test was performed following a seeding diagram that consisted of making the wells on the agar infected with the microorganism. The necessary amount of controls and samples were placed with a micropipette in each of them. Chloramphenicol was used as a positive control, and dimethylsulfoxide was used for negative control. The boxes were left in the 37°C incubators for 24 hours; after this period, the inhibition halos (mm) were read. Each determination was performed in triplicate, and the inhibition halos were determined. Based on the inhibition halos, the percentage of Inhibition was calculated to identify which fraction or extract had the highest antimicrobial activity. To find this percentage, the following statistical formula (Eq .1) was used:

$$\%\ of\ Inhibition = \frac{Halo\ Extract - Halo\ lank}{positive\ control\ halo - Halo\ lank} \times 100 \ldots (Eq\ 1)$$

2.4.3 Antifungal evaluation

The conditions for the test with *Candida* sp were similar to those performed for bacteria, the suspension of this fungus was prepared, and potato dextrose agar (PDA) and Mueller Hinton agar were used. Clotrimazole (98%) at a 40 mg/mL concentration was used as a positive control and DMSO as a negative control. The samples were analyzed in triplicate and the seeding diagrams proposed for the bioassay were followed. Subsequently, the boxes were left in the incubator at 27°C for five days and after this time, the inhibition halos were measured [14].

2.4.4 Minimum Inhibitory Concentration (MIC)

This test was performed to determine the lowest concentration of the extract or fraction that inhibits the growth of the microorganism. The butanol fraction was used since it was the one with the highest percentage of Inhibition against *S.aureus*, which was determined after carrying out the different bioassays. Successive dilutions of the butanol fraction were prepared, starting from a stock solution of 30 mg/mL followed by concentrations of 15 mg/mL, 7.5 mg/mL, 3.75 mg/mL, 1.875 mg/mL and 0.9375 mg/mL in one volume of 2 µL of the butanol fraction. A 3 mL multiwell plate was used and 2 ml of MH agar and 2 µL of the *S. aureus* suspension were added to each well. The seeding was distributed on the plate as follows: one well as medium control (CM), that is, it does not contain a suspension of the microorganism or the butanol fraction, positive control (Chloramphenicol) (+), negative control (DMSO) (-) and bacterial growth control (CCB) and each of the dilutions of the butanol fraction was applied in triplicate.

3.0 RESULTS AND DISCUSSION

The crude extract obtained by maceration of 450g of the aerial parts of *E.serpens* weighed 42.3 g, which corresponds to a yield of 9.4%.

3.1 Obtaining the fractions from the crude extract: 12.098 g of the crude extract was fractionated by Soxhlet extraction, and the quantities obtained for each fraction are listed in Table 1.

Table 1. Quantity of the fractions obtained from the fractionation of the crude extract with solvents

Fraction	Weight (g)	Yield %
EtOH	0.92	7.60
Bu-OH	2.90	23.97
n-hexane	5.80	47.94
$CHCl_3$	2.02	16.70

3.2 Preliminary Phytochemical Analysis: According to the results of the different phytochemical tests carried out, it was identified that the crude extract of *E.serpens* presents compounds such as alkaloids, triterpenoids and/or steroids, saponins, phenolics, tannins, flavonoids and quinones. Where it was observed that the most abundant compounds are: triterpenoids and/or steroids, saponins and tannins. The results obtained from the preliminary phytochemical analysis are presented in Table 2. Regarding the physical-chemical characteristics of the crude extract, a pH of 5.55 could be established; the refractive index of 1.38; Relative density of 0.84 g/mL and total solids of 6.28%. The results obtained in the preliminary test are summarized in Table 2.

Table 2: Phytochemical triage of crude extract of *E.serpens*

Metabolites	Rehearsal	Crude or Ethanolic Extract
alkaloids	Dragendorff	++
alkaloids	Wagner	+++
glycosides/cardio tonics	Bouchet	-
Triterpenoids and/or steroids	Liebermann–Burchard	+++
saponins	Foam	+++
Tannins/phenolics	$FeCl_3$	+++
Tannins	Gelatin-salt	+ + +
Flavonoids	Shinoda	+ +
Flavonoids	Leukoanthocyanidins	-
Quinones	Acid behaviour and electron donor	+ +
steroids	Vanillin	-
coumarins	Erlich	-

Conventions: (+++) abundant; (++) fair; (+) doubtful; (-) negative

3.3 Toxicity Tests

The dose or concentration of the evaluated extract was defined as the independent variable, the ratio of dead/exposed individuals as the dependent variable, and the sample size of 10 exposed organisms. This program established the regression (Figure 2) and allowed the calculation of the dose that causes the mortality of 50% of the population that corresponds to the lethal concentration fifty (LC_{50}) that for the *E.serpens* extract was 135.31 µg/mL, which according to what was reported by [11] This extract is moderately toxic ($100<LC_{50}<1000$).

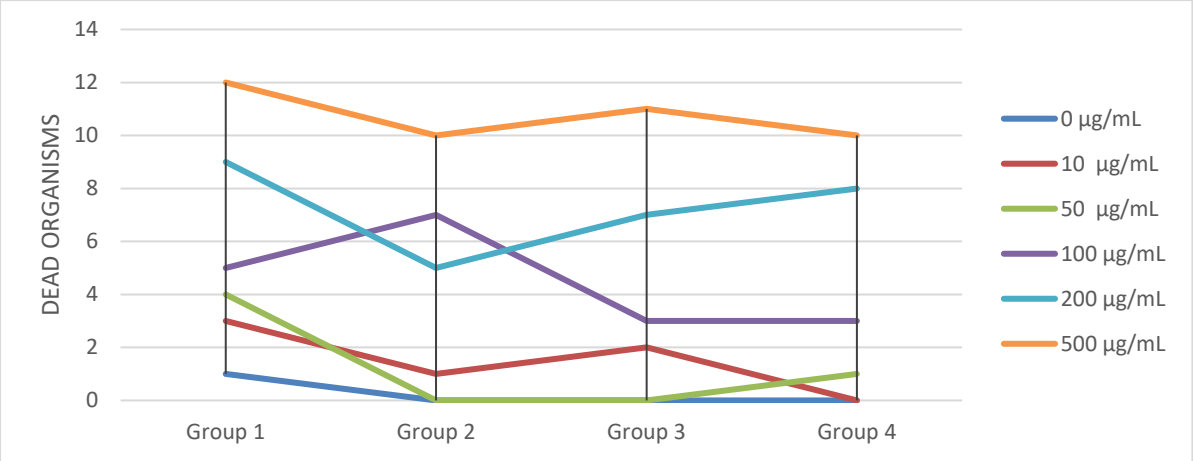

Figure 2. Probit (Dead/exposed) with 95% confidence interval Dose Vs Dead organisms

3.4 Biological Activity Assays: Initially, the yeast *Candida* sp was visualized microscopically under Gram stain, where yeasts of different sizes, round or oval. For the evaluation of the antimicrobial activity of the crude extract and the fractions (ethanol, butanol, chloroform and n-hexane) against the different microorganisms, the test conditions were initially established:

- Dimethyl sulfoxide (DMSO) was used as a solvent for the samples used, where up to 50 µL can be used without Inhibiting the microorganism being observed.
- Different concentrations were evaluated for the negative control (DMSO) (C-) from 75 µg/mL up to the use of a final concentration of 20 µg/mL. In this, the growth of the microorganism was according to the petri dish used for the trial.
- The concentration of the positive control was initially evaluated at 75 µg/mL, but at that concentration, the diameter of the inhibition halos was huge, which flooded the negative controls. Due to this, several tests were carried out, decreasing the concentration until reaching the optimal value of Clotrimazole (40 mg/mL) and chloramphenicol (0.5 mg/mL).
- The samples were prepared at a concentration of 30 mg/mL, and two test volumes, ten and 20µL, were evaluated, establishing that there was more excellent reproducibility and repeatability of the values corresponding to the inhibition halos. Initially, test trials were carried out with the different microorganisms to identify which had an inhibitory effect. For the interpretation of the results, the test indicated for the inhibition percentage was taken as a reference, where an antimicrobial action is considered high when its relative inhibition percentage is more significant than 70%, intermediate between 50-70% and low when it is less than 50%.[14].

Table 3 shows the results obtained in the bioassays of the crude extract and the fractions (ethanol, butanol, chloroform and hexane) against each microorganism evaluated.

Table 3. Preliminary results of the antimicrobial activity of the extracts and fractions of *E.serpens* against the microorganisms *S. aureus*, *Candida* spp., *E. coli* and *Klebsiella* spp.

Extract	*S.aureus*	*Candida* spp	*E.coli*	*Klebsiella* spp
Crude	++	+	-	-
Et-OH	++	+	-	-
Bu-OH	++	+	-	-
n-Hexane	++	+	-	-
Chloroform	++	+	-	-

Conventions: (+++) high activity; (+) intermediate activity; (-) null activity.

In the biological activity tests, it was observed that the extract and the evaluated fractions present high activity against *S. aureus* and an intermediate activity against *Candida* sp. While against *E. coli* and *Klebsiella* sp, no activity was evidenced.

The results obtained for the crude extract and the fractions of *E.serpens* with *E. coli* and *Klebsiella* sp may be because these Gram-negative bacteria can generate changes in the lipid bilayer. However, the membrane's permeability is altered mainly by changes in the porins. Porins are proteins that form water-filled channels embedded in the outer membrane that regulate the entry of some elements, including the components of the extract. Changes in its conformation can lead to the outer membrane not allowing the passage of these components to the periplasmic space [14-15].

Based on these preliminary results, the tests were carried out to evaluate the potential of the extract against *S. aureus* and *Candida* sp. The tests were carried out in triplicate to establish the percentages of Inhibition and the minimum inhibitory concentration of the most active microorganism. The results obtained from the bioassays with each microorganism studied are shown below**.**

3.5 Evaluation of the antibacterial activity of the extract and its fractions of *E.serpens* against *Staphylococcus S. aureus and Candida spp*

The bioassay results are shown in Table 4 and figure 3 where it was observed that the crude extract and the fractions present activity against this bacterium. The crude extract presented an inhibition percentage of 50.24%. In contrast, a higher inhibition percentage was observed in the fractions, as was the case of the butanol fraction, which was even higher than that of the positive control with 110.23% and the hexane fraction with an inhibition percentage of 81.36%, the chloroform fraction with 65.27% and the ethanolic fraction with 33.25% at a volume of 20 µL. According to the reference test, it is considered that the butanol fraction has high antibacterial action. In contrast, the hexane fraction acts intermediately and the ethanolic and chloroform fractions produce a low antibacterial action.

On the other hand, the butanol fraction presented an inhibition percentage comparable to the positive control (chloramphenicol), which obtained 100% sensitivity. The statistical analysis of the data theoretically presents a coefficient of variation less than 5% and not greater than 10% [10] in terms of the statistical results obtained, a percentage less than 17% were presented for all fractions of the extracted oil analyzed, which reports that there were failures in the handling of the personnel during the test, for the butanol fraction (2.2%). Hexane fraction (4%) presented reliable data since the CV was less than 5%.

Table 4. Bioassays at a volume of 20 µL injected from the extract, fractions and controls against *S. aureus & Candida spp*.

	Extract	Coding	Inhibition: Halos (mm) R-1	R-2	R-3	Avg.	SD	Coefficient of variation (CV)	% of Inhibition
S.Aureus	Crude	Ext	9	10	13	10.67	1.70	2.89	50.24
	Et-OH	Fr-1	7	9	9	8.33	0.94	0.89	33.25
	Bu-OH	Fr-2	23	23	23	23.00	0.00	0.00	110.23
	n-Hexane	Fr-3	7	6	7	6.67	0.47	0.22	81.36
	Chloroform	Fr-4	15	17	15	15.67	0.94	0.89	65.27
	Positive control	C+	23	20	18	20.33	2.05	4.22	-
	negative control	C-	0	0	0	0.00	0.00	0.00	-
Candida spp	Crude	Ext	7	7	7	7.00	0.00	0.00	26.35
	Et-OH	Fr-1	7	8	7	7.33	0.47	0.22	30.25
	Bu-OH	Fr-2	0	0	0	0.00	0.00	0.00	0
	n-Hexane	Fr-3	2	3	4	3.00	0.82	0.67	12
	Chloroform	Fr-4	0	0	0	0.00	0.00	0.00	0
	Positive control	C+	3	3	3	3.00	0.00	0.00	-
	negative control	C-	0	0	0	0.00	0.00	0.00	-

Conventions: Replica 1 (R1), Replica 2 (R2), Replica 3 (R3)

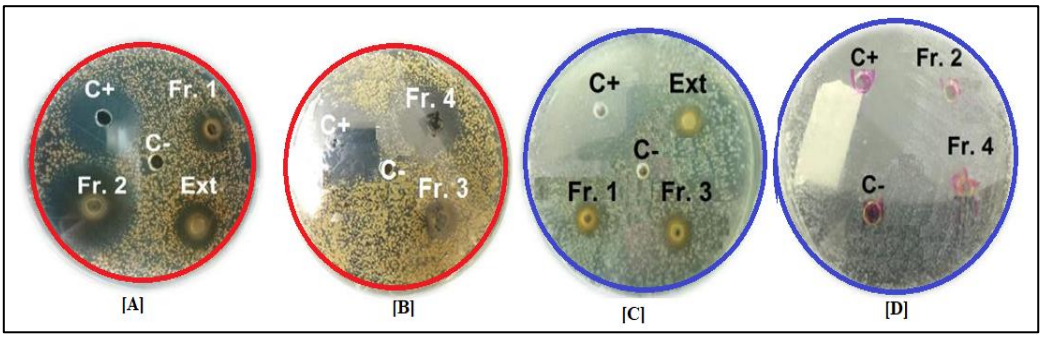

Figure 3. Evaluation of the activity of the extract and its fractions of *E.serpens* against *S. aureus* [A]. The crude extract (Ext), the ethanolic fraction (Fr. 1), butanol fraction (Fr. 2). [B]. Chloroform fraction (Fr. 3) and hexanic fraction (Fr. 4). Positive control (C+), Negative control (C-) & fractions of *E.serpens* against *Candida* sp. [C] crude extract (Ext), ethanolic fractions (Fr. 1), chloroform (Fr. 3). [D]. butanolic (Fr. 2), and hexanic (Fr. 4). Positive control (C+), Negative control (C-).

3.6 Minimum Inhibitory Concentration

This test was performed with the fraction in n-butanol because it was the one that presented the highest percentage of Inhibition. The test was carried out by two methods: dilution in MH agar and nutrient broth. In the first, Inhibition of the microorganism was observed at the concentration of 30 mg/mL, while with the other dilutions, growth of the microorganism was observed; as for the positive control, no microbial growth was detected, as shown in Figure 4, where the Inhibition against *S. aureus* was lower at concentrations of 16.5 mg/mL, 7.6 mg/mL, 3.78 mg/mL, 1.88 mg/mL, 0.94 mg/mL. Regarding the method of dilution in broth, decreasing concentrations of the butanol fraction were placed in tubes with nutrient broth; these concentrations were the same as those used in the first method; at the end of the test, it was observed that the tubes presented insolubilization because the test was performed incorrectly. Therefore, this method was discarded from the study.

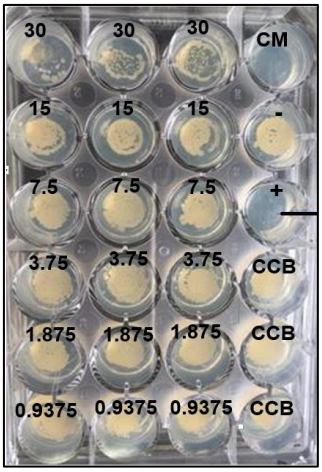

Figure 4. Schematic of the minimum inhibitory concentration (MIC) assay; Medium control (CM); Negative Control (-); Positive Control (+); Bacterial Growth Control (CCB); All concentrations were in milligrams (mg).

4.0 CONCLUSION

In a preliminary phytochemical examination, it was discovered that the crude extract of the plant species *Euphorbia serpens* Kunth contains secondary metabolites, which were identified by the use of different colour and precipitation tests. In the toxicity test with *Artemia salina*, it was concluded that for the crude extract of *E.serpens*, the LC_{50} was 135.31µg/mL, thus presenting a moderate degree of toxicity, which indicates that studies can be carried out about the possible Applications. The butanolic fraction of *E.serpens* showed high activity (greater than 70%) against the bacteria *S. aureus*; the crude extract and the hexane fraction showed intermediate activity (50%-70%), while the ethanolic and chloroform fractions showed lower activity. The MIC determined for the butanol fraction was 30 mg/ml, which indicates that the minimum Inhibition against *S. aureus* was observed at that concentration. The crude extract and the ethanolic fraction showed moderate antifungal

activity against the yeast *Candida spp*, obtaining inhibition percentages of 26.35 % and 30.25 %, respectively.

REFERENCES

[1]. Zeghad, Farida & Djilani, Salah Eddine & Djılanı, Abdelouaheb & Dıcko, Amadou. (2016). Antimicrobial and Antioxidant Activities of Three Euphorbia Species. *Turkish Journal of Pharmaceutical Sciences.* 13. 22-37. 10.5505/tjps.2016.29491.

[2]. Lin J, Dou J, Xu J, Aisa HA. Chemical composition, antimicrobial and antitumor activities of the essential oils and crude extracts of *Euphorbia macrorrhiza*. *Molecules*. 2012, 3;17(5):5030-9.

[3]. Mahady, Gail. (2005). Medicinal Plants for the Prevention and Treatment of Bacterial Infections. *Current pharmaceutical design*. 11. 2405-27. 10.2174/1381612054367481.

[4]. Dahiya, Praveen, and Sharmishtha Purkayastha. "Phytochemical Screening and Antimicrobial Activity of Some Medicinal Plants against Multi-drug Resistant Bacteria from Clinical Isolates." *Indian journal of pharmaceutical sciences,* Vol. 74.5 (2012): 443-50. doi:10.4103/0250-474X.108420

[5]. Wolf, M & Király, Gergely. (2014). *Euphorbia serpens* (Euphorbiaceae) is a new alien species in Hungary. *Acta Botanica Hungarica*. 56. 260. 10.1556/ABot.56.2014.1-2.16.

[6]. Hügin, G. (2008). *Chamaesyce Serpens* subsp. fissistipula -Neubewertung von *Euphorbia Serpens var. fissistipula* (Euphorbiaceae). *Feddes Repertorium*. 109. 509-519. 10.1002/fedr.19981090705.

[7]. Ardenghi, Nicola & Mossini, Sara. (2015). Notula 254. *Euphorbia serpens Kunth*. In: Galasso G., Nepi C., Domina G. & Peruzzi (Eds.). Notulae alla Flora esotica d'Italia 12 (244-287). *Informatore Botanico Italiano*. 47. 80.

[8]. *Euphorbia serpens Kunth* in A. von Humboldt et al., *Nov. Gen. Sp*. 2(fol.): 41; 2(qto.): 52. 1817.

[9]. Sanabria-Galindo, A. (1983) Preliminary Phytochemical analysis: methodology and application in evaluating 40 plants of the Compositae family. Department of Pharmacy, Faculty of Sciences, National University of Colombia, Colombia pp. 61-86

[10]. Rajeh, M.A.B.; Zuraini, Z.; Sasidharan, S.; Latha, L.Y.; Amutha, S. Assessment of *Euphorbia hirta* L. Leaf, Flower, Stem and Root Extracts for Their Antibacterial and Antifungal Activity and Brine Shrimp Lethality. *Molecules* 2010, 15, 6008-6018. https://doi.org/10.3390/molecules15096008.

[11]. Logarto Parra A, Silva Yhebra R, Guerra Sardiñas I, Iglesias Buela L. Comparative study of the assay of *Artemia salina* L. and the estimate of the median lethal dose (LD_{50} value) in mice, to determine acute oral toxicity of plant extracts. Phytomedicine. 2001 Sep;8(5):395-400. DOI: 10.1078/0944-7113-00044. PMID: 11695884.

[12]. Jaramillo Jaramillo C, Jaramillo Espinoza A, D'Armas H, Troccoli L, Rojas de Astudillo L. (2016), Concentrations of alkaloids, cyanogenic glycosides, polyphenols and saponins in selected medicinal plants from Ecuador and their relationship with acute toxicity against *Artemia salina*. *Rev Biol Trop*. Sep;64(3):1171-84. Spanish. PMID: 29461791.

[13]. Flanagan, J. N., & Steck, T. R. (2017). The Relationship Between Agar Thickness and Antimicrobial Susceptibility Testing. *Indian journal of microbiology*, 57(4), 503–506.

[14]. Liu, Y., Tortora, G., Ryan, M. E., Lee, H. M., & Golub, L. M. (2002). Potato dextrose agar antifungal susceptibility testing for yeasts and molds: evaluation of phosphate effect on antifungal activity of CMT-3. *Antimicrobial agents and chemotherapy*, 46(5), 1455–1461.