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NATURAL PRODUCTS

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Microbiological evaluation and chemical composition of organic extracts from Euphorbia aff. viridis (Klotzsch & Garcke) Boiss on Staphylococcus Aureus, Klebsiella Pneumoniae and Escherichia Coli

Evaluación microbiológica y composición química de extractos orgánicos de Euphorbia aff. viridis (Klotzsch & Garcke) Boiss sobre Staphylococcus Aureus, Klebsiella Pneumoniae y Escherichia Coli

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Resumen

Euphorbia aff. viridis (Klotzsch Garcke) Boiss. es una especie vegetal perteneciente a la familia *Euphorbiaceae*, forma parte de las más diversas del continente americano, y es empleada en múltiples tratamientos en medicina ancestral. En el presente estudio se analizaron los compuestos orgánicos extraídos de las hojas de la planta. Se estableció la descripción y clasificación taxonómica de la especie vegetal. Para el estudio fitoquímico se analizaron los fenoles, taninos, saponinas, cumarinas, lactonas y flavonoides, observándose resultados positivos para cada compuesto químico. La caracterización química mediante cromatografía de gases acoplada a espectrometría de Masas GC-MS, mostró $36,66\,\%$ de lanosterol, $12,25\,\%$ β-sitosterol, $5,11\,\%$ $3-\beta-colest-4-en-3-ol$, $4,73\,\%$ ácido hexadecanoico como elementos mayoritarios. El ensayo microbiológico de extractos etanólicos mostró un porcentaje de inhibición del $44\,\%$ sobre Klebsiella pneumoniae (ATCC70693) y $43\,\%$ en Escherichia coli (ATCC10536) con una CMI de $30\mu l/ml$ para cada cepa, y se observó un resultado negativo de inhibición para Staphylococcus aureus (ATCC24213). Los extractos orgánicos de *Euphorbia aff. viridis (Klotzsch Garcke) Boiss.* presentaron actividad antimicrobiana pudiéndose observar su potencial uso como agentes antimicrobianos.

Palabras clave: Antimicrobiano, extracto orgánico, GC-MS, Euphorbia aff. viridis (Klotzsch Garcke) Boiss.

Abstract

Euphorbia aff. viridis (Klotzsch Garcke) Boiss. a plant species belonging to the *Euphorbiaceae* family, is one of the most diverse of the American continent, and is used in multiple treatments in ancestral medicine. In the present study, organic compounds extracted from the leaves of the plant were analyzed. The description and taxonomic classification of the plant was established. For the phytochemical study, phenols, tannins, saponins, coumarins, lactones and flavonoids were analyzed, and positive results were observed for each chemical compound with the exception of alkaloid compounds. The chemical characterization by gas chromatography coupled to mass spectrometry GC-MS, showed 36,66% lanosterol, 12,25% β-sitosterol, 5,11% $3-\beta-cholest-4-en-3-ol$, 4,73% hexadecanoic acid as major elements. The microbiological assay of ethanolic extracts showed a 44% inhibition percentage on Klebsiella pneumoniae (ATCC70693) and 43% on Escherichia coli (ATCC10536) with an MIC of $30\mu l/ml$ for each strain, and a negative inhibition result was observed for Staphylococcus aureus (ATCC24213). The organic extracts of *Euphorbia aff. viridis (Klotzsch Garcke) Boiss.* presented antimicrobial activity being able to observe their potential use as antimicrobial agents.
Keywords: Antimicrobial, organic extract, GC-MS, Euphorbia aff. viridis (Klotzsch Garcke) Boiss.

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1 Introduction

In the Ecuadorian population, the use of plants for therapeutic purposes is closely linked to cultural traditions. Nowadays, ethnobotany has awakened a consciousness of change in the scientific and academic generations, studies carried out by Coy and Castiblanco (2016), describe the medicinal importance, taxonomy and ethnobotanical uses of species of the genus *Euphorbiaceae*. The Euphorbiaceae family comprises about 8 100 species, common in tropical countries, being part of the most diverse families among the Magnolophytas, after the Orchidaceas, Asteraceae, Fabaceae, Poaceae and Rubiaceae (Ogbulie and Anyanwu, 2007; Zegarra, 2015). This family is one of the angiosperms with more diversity in terms of the habitat and morphology, they vary in size from the trees as the Havea with high altitude to smaller plants such as cactus (Al-Mughrabi, 2003; Coy and Castiblanco, 2016). According to ethnobotanical studies conducted by Mwine and Van Damme (2011) and Oyerma and Opio (2010) Euphorbiaceae family is employed for medicinal purposes, such as the species *Hura crepitans L.*, which is characterized by possessing astringent characteristics.

In the ancestral medicine, the use of species of the Euphorbiaceae family is varied, being used in dermatology, as fungicide and antimicrobial, as treatment of gastrointestinal diseases, and for bacterial diseases such as gonorrhea (Azuaje and Rodríguez, 2017). Studies carried out by Sabandar and Sahidin (2013) on the genus *Jatropha* have described its use in skin diseases such as infections and inflammations.

Euphorbia aff. viridis (Klotzsch & Garcke) Boiss. is a phanerogam plant that multiplies asexually through stakes (Giraldo and Polanco, 2015). Studies carried out by Flores and Lazo (2012) indicate that their sowing is done when the moon starts its phases (crescent moon or full moon), due to a greater incidence of the magnetic fields on the root system of the plant; and depending on each lunar phase the influence on the stems and their foliage will be greater until the final development of their inflorescence. Once this stage is completed, the harvest is made in full moon, since the parts that make up the plant will be found with a higher percentage of each of its constituent chemical compounds, being able to use potentially in traditional medicine (Torres, 2012).

The research and contributions made in Ecuador on *Euphorbia aff. Viridis (Klotzsch & Garcke) Boiss.* with regard to their medicinal properties are scarce. Studies carried out by Bittner and Silva (2001) showed that *Euphorbia peplus, Euphorbia lactiflua, Euphorbia portulacoides and Euphorbia serpens* have antimicrobial activity, being indicative for *Euphorbia aff. Viridis (Klotzsch Garcke) Boiss.* to have chemical compounds with similar properties related to the described species.

In this study, it was proposed to relate the therapeutic properties of the extract with the biological activity against microbial agents (Zampini and Isla, 2007), for that reason, different techniques were used for the phytochemicals analysis and antimicrobial activity (López et al., 2016), on *Staphylococcus aureus* (ATCC24213), *Klebsiella pneumoniae*(ATCC70693) and *Escherichia coli*(ATCC10536). Also, the compounds present in *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* were analyzed; as well as studies by Suzuki et al. (2006), giving the necessary guidelines for the exploitation of this vegetal species that grows in Ecuador, for its later use in ancestral medicine.

2 Materials and methods

The taxonomic identification of the species was carried out on August 9, 2017, by means of a curator of the herbarium of Universidad de Azuay (Figure 1), in Cuenca, Ecuador.

Figure 1. Plant *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* with flower.

2.1 Collection of the plant material

The leaves of the plant *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* were collected in Cuenca, Azuay Province, Ecuador (Long:-79.0093518 Lat:-289014096), by using Stanley Steel pruning shears; subsequently, these were collected in isopropylene containers and transported to the Life Sciences Laboratories of Universidad Politécnica Salesiana.

2.2 Obtaining of the extracts

The fresh leaves of *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* were dried at $60°C$ for eight (8) days, and it was possible to obtain fine particles by mechanical pressure. The amount of vegetable matter used was $50g$ for dry extract as for fresh extract. Subsequently, $10ml$ of extract was obtained by macerating it with absolute ethanol, eliminating the solvent and using a rotary evaporator TECNAL TR-211 (Hernández et al., 2014; González Villa, 2004; Muhammad Abubakar, 2009).

2.3 Phytochemical analysis

According to López et al. (2017) the determination and presence of phenols and tannins, saponins, coumarins and lactones, flavonoids, quinones and alkaloids was done in the extract obtained.

2.4 Gas chromatography-GC-MS mass spectrometry

An agilent model GC-6890 gas chromatograph was used coupled to a quadrupole HP 5973N mass spectrometer. The injection of the sample was carried out by the "split"mode with a ratio of 1:10 (Parrales and William, 2012), with the temperature of the injector at $280°C$. It was worked with an initial temperature of $80°C$, increasing up to $310°C$, followed by an isothermal process of $20mn$ (García and Castro, 2010). The final injection volume was 1 . The sample components were fractioned in the HP-5MS column. The total elution time was 100 minutes, operated by electronic ionization at $70eV$ with a mass range of $35 - 700uma$. As carrier gas, helium was used at a flow rate of $0{,}8ml/min$.

2.5 Microbiological study

The diffusion technique in Agar was used using discs, based on the guidelines and protocols established by Clinical Laboratory Standard Institute (CLSI) and the World Health Organization (WHO). There is a basis in the relationship between the concentration of the antibiotic to be used and the formation of inhibition halos produced on the surface of the plaque containing the culture medium (Ramírez and Vargas, 2013). Dried leaf extract and fresh leaf extract were used in concentrations of pure extract (100 %), 80/20 and 50/50 ethanol-water.

2.6 Evaluation of antimicrobial activity

$0{,}03g$ of the crude extract was weighed and was solubilized with $1ml$ of dimethylsulfoxide (DMSO) of analytical grade to achieve a concentration of $30mg/ml$. For the activation of the microorganisms: *Staphylococcus aureus* (ATCC24213), *Klebsiella pneumoniae* (ATCC70693) and *Escherichia coli* (ATCC10536), the bacteria were isolated in nutritive agar of Mark Acumedia, prepared according to specifications of the company and Autoclaved at $121°C$ and 1 atmosphere for 15 min.
The procedure was performed using the depletion technique of the sample by Striae, in Petri dishes, incubated during $18 - 24$ hours at $37°C$. Once the incubation time was completed, with the help of a sterile bacteriological loop, 3 to 5 morphologically similar colonies were taken and suspended in a saline NaCl solution at 0,9 % until the turbidity comparable to the McFarland 0,5 pattern was reached, in a qualitative way.
Muller-Hilton agar brand Difco^{TM} was used for the test of antibacterial activity. It was placed in Petri dishes, which were impregnated with $100\mu l/box$ with the adjusted suspension of each indicator bacterium, sterile discs of $6mm$ of diameter were used, moistened with $10\mu l$ of diluted extract; chloramphenicol was employed as positive control in concentration of $100\mu l/ml$, and as negative control DMSO was employed. Petri dishes were then incubated at $37°C$ for 24 hours; each of the trials was carried out in triplicate (Hammer and Riley, 1999).

The inhibition percentage was calculated by means of the formula (1) taking as reference the measurement of the diameter of the inhibition area of the positive control and the halo measurement of the extracts tested (Corzo, 2012).

El porcentaje de inhibición se calculó mediante la fórmula (1), teniendo como referencia la medición del diámetro de la zona de inhibición del con-

trol positivo y la medición del halo de los extractos testeados (Corzo, 2012). Where ⊘ extract halo, is the diameter of the halo formed by the disc containing the extract; ⊘ white halo, is the diameter of the halo formed by the disc of the negative control and ⊘ positive control halo, is the diameter of the halo formed by the disc with the positive control.

$$\%\text{Inhibition} = \frac{(\oslash\text{extract halo} - \oslash\text{white halo})}{(\oslash\text{positive control halo} - \oslash\text{white halo})} \times 100 \quad (1)$$

2.7 Determination of the minimum inhibitory concentration (CMI)

The minimum inhibitory concentration (CMI) was applied to determine the lowest concentration of each of the extracts that inhibits the growth of the bacteria. The endpoint (CMI) is considered the lowest concentration of compound on which the microorganism tested does not present visible development (Cruz and Rodríguez, 2010). The CMI was evaluated only for extracts that showed inhibition against the reference bacteria. It was carried out using the dilution method in TSB agar (Acumedia brand), where the extract was incorporated to be evaluated in the medium with agar. The technique was carried out in micro plates with flat bottom of 24 wells, in which different solution volumes of the extracts were laid out, and micro dilutions were performed with the culture medium.

The concentrations tested were $30\mu l/ml$, $15\mu l/ml$, $7{,}5\mu l/ml$, $3{,}75\mu l/ml$, $1{,}875\mu l/ml$ and $0{,}9375\mu l/ml$ for *K. pneumoniae*, and from $60\mu l/ml$ for *E. coli*. In each well, $2\mu l$ of *Klebsiella pneumoniae* (ATCC70693) and *Escherichia coli* (ATCC10536) were sown at a concentration of $1{,}5 \times 106$ UFC/ml, affordable to tube 0.5 in the McFarland turbidity scale. After 24 hours of incubation at $37°C$, the microplates were visually examined.

3 Results

The amount obtained from dry extract was $14{,}82ml$ and fresh extract $20{,}44ml$. In Table 1, the phytochemicals screenings corresponding to the extract obtained from *Euphorbia aff. Viridis (Klotzsch & Garcke) Boiss.* and 100 % ethanol solution are shown. In Table 2, the results of the presence of alkaloids in the analyzed extract are collected. The analysis of gas chromatography coupled with GC-MS mass spectrometry was able to show the results shown in Table 3.

Table 1. Phytochemicals Screening Results.

Components	Resuls
Trial with phenols and tannins	Positive
Trial with coumarins and lactones	Positive
Trial of quinones	Positive
Trial of saponins	Positive
Trial of catechins	Positive

Table 2. Results of the Alkaloid Test

Essay	Results
Dragendorff Essay	Negative
Wagner Essay	Negative
Mayer Essay	Negative

Table 3. Results of the mass chromatography test.

No.	Tr	Relative abundance %	Compound
1	5722	0.92	Benzoic acid
2	6623	0.52	Glycerol
3	21499	1.46	Ethyl ether of hexadecanoic acid
4	22547	4.73	Hexadecanoic acid
5	24905	10.40	3,7,11,15-tetramethyl-2-hexadecene
6	25417	0.81	9, 12-Octadecadienoic acid
7	25529	2.26	α-Linoleic Acid
8	26006	1.20	Octadecadienoic acid
9	38944	5.49	Unknown
10	40298	36.66	Lanosterol
11	40852	13.72	Lanosterol (isomer)
12	41213	12.15	β-sitosterol
13	41608	2.91	β-amyrin
14	41872	1.66	Cycloartenol
15	42248	5.11	3-β-colest-4-in-3-ol

*Tr: Retention time in the column.

The antibacterial analysis for the isolated microorganisms: *Staphylococcus aureus* (ATCC24213), *Klebsiella pneumoniae* (ATCC70693) and *Escherichia coli* (ATCC10536), are shown in Table 4. The study was carried out based on the extract obtained from fresh and dry leaves in ethanol solution, resulting that dry leaves presented greater antimicrobial activity compared to the extract of fresh leaves. The extracts studied showed inhibition against *Klebsiella pneumoniae* (ATCC70693) and *Escherichia coli* (ATCC10536) being negative for *Staphylococcus aureus* (ATCC24213), having better results in the extracts of dry leaves.

Table 4. Results before the antimicrobial activity test of the ethanolic extract.

		100.00 %	80/20	50/50
Staphylococcus aureus	Dry	(-)	(-)	(-)
(ATCC 24213)	Fresh	(-)	(-)	(-)
Klebsiella pneumoniae	Dry	(+)	(+)	(-)
(ATCC 70693)	Fresh	(+)	(-)	(-)
Escherichia coli	Dry	(+)	(+)	(-)
	Fresh	(+)	(-)	(-)

(+) Positive result, (-) Negative result.

In Figures 2 and 3, the inhibition of the extract against the microorganisms used is exposed in a percentage way, and it is calculated according to the inhibition halo, and having as reference the positive control. Following the protocol proposed by Corzo (2012), the CMI was determined at 24 hours corresponding to the observation time, taking into consideration that the inhibition is carried out at a concentration of $30\mu l/ml$in both studies. Figures 4 and 5 show the results obtained from CMI against *K. pneumoniae* and *E. coli*, respectively.

La Granja: *Revista de Ciencias de la Vida* 29(1) 2019:119-129.

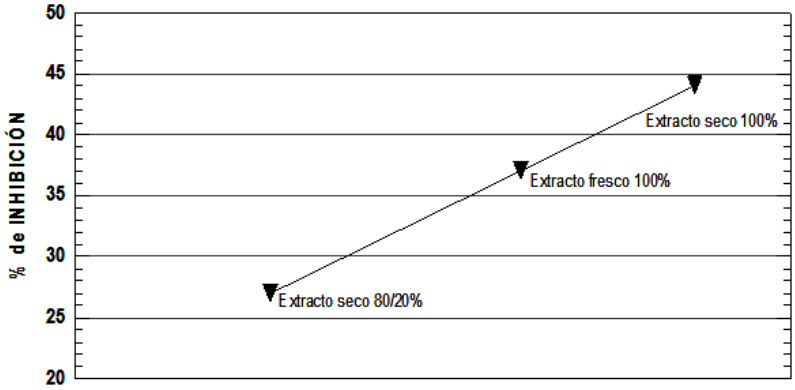

Figure 2. Inhibition percentage of ethanol extracts versus *Klebsiella pneumoniae*.

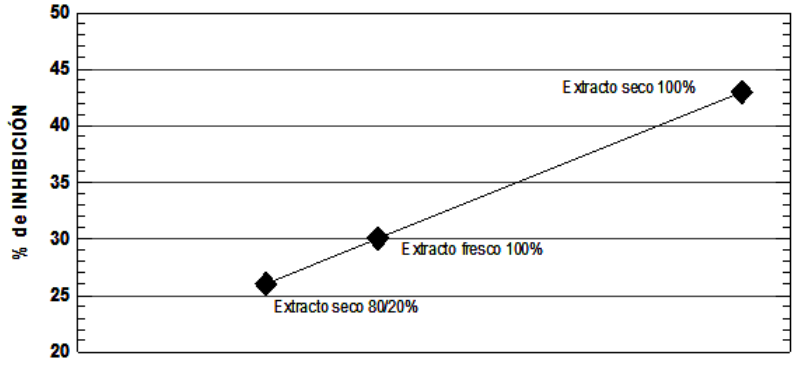

Figure 3. Inhibition percentage of ethanol extracts against *Escherichia coli*.

4 Discusión

The alcoholic extract of dry and fresh leaves of *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* confirmed the presence of phenols, tannins, coumarins and lactones, quinones, saponins and catechins, giving positive control to the corresponding phytochemicals analysis, and being similar with the studies carried out by Gutiérrez and Montes de Oca Porto (2011), where Lactones, phenols, coumarins and tannins were isolated from hexanic extracts and ethyl acetate extract. On the other hand, in the "phytochemicals stud of leaves from the vegetal species *Croton schiedeanus (Euphorbiaceae)*çarried out by Chiappe (2015), it was possible to demonstrate the presence of secondary metabolites of type carotenoid, phenolic, flavonoid and alkaloid, agreeing with the chemo family and gender taxonomy.

The presence of alkaloids was not detected in this study in relation to several species studied of the genus, whose result has been positive, as in Bittner and Silva (2001), where they express that alkaloids of the morphine and aporphine type were isolated. Gomes and Leite (2017), conducted a study on "physicochemical characteristics and cytotoxic effect of methanol extract of *Croton heliotropiifolius Kunth (Euphorbiaceae)*", where the physical-chemical analysis performed by TLC, HPLC, FT-IR and UV-Vis, revealed the presence of flavonoids and the absence of alkaloids, coumarins, saponines and condensed tannins.

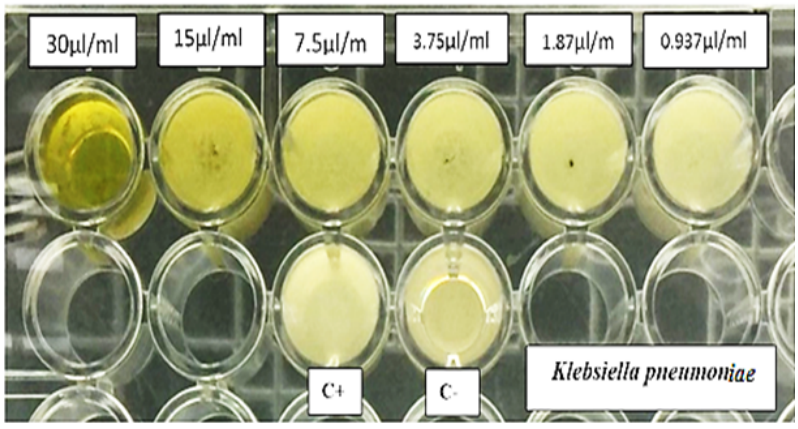

Figure 4. Result of the minimum inhibitory concentration against *Klebsiella pneumoniae.*

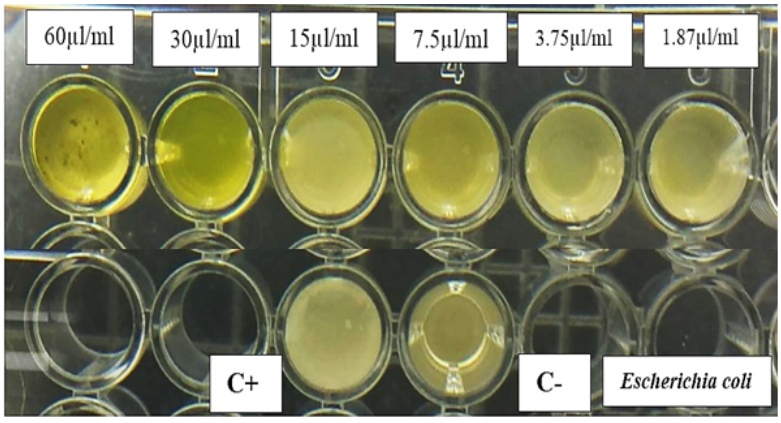

Figure 5. Result of the minimum inhibitory concentration against *Escherichia coli.*

GC-MS is a very robust method for analyzing the chemical molecules present in a plant species, being able to establish their majority compounds as well as possible chemical markers for the species of interest (Valdéz and Ramírez, 2018). Regarding the analysis of GC-MS *Euphorbia aff. viridis. (Klotzsch Garcke) Boiss.*, it was possible to identify the presence of benzoic acid 0,92 %, glycerol 0,52 %, hexadecanoic acid ethyl ester 1,46 %, hexadecanoic acid 4,73 %, 3,7,11,15-tetrametill-2-hexadecenol 10,40 %, acid-9.12-octadecenoic acid 0,81 %, linoleic acid-α -2,26 %, octadecanoic acid 1,20 %, lanosterol 36,66 %, lanosterol (Isomer) 13,72 %, β-sitosterol 12,15 %, β-amirine 2,91 %, cycloartenol 1,66 %, 3-β-Coles-4-in-3-ol 5,11 %, Being lanosterol the majority compound with 36,66 %.

Rodríguez Pava and Sánchez Leal (2017), in .ªntimicrobial activity of four plant varieties against the clinical importance of pathogens in Colombia", conclude that flavonoids and quinones have important antibacterial properties, that with the contact with Streptococcus pneumoniae and Staphylococcus aureus have shown activity on these strains, being null for *Staphylococcus aureus* (ATCC24213) in this study.

Studies conducted by Alvarez and Toso (2018), on the species *Euphorbia peplus* showed positive results for the antimicrobial activity against *S. aureus*, while *Euphorbia serpens* is effective against *E. coli* and *S. aureus*, being similar with the results obtained in this study in which bioactivity of the extract was observed at 100 % on *Escherichia coli*. Corzo (2012) and Ogbulie and Anyanwu (2007), show that

the family of the *Euphorbiaceas* possesses antimicrobial activity, being able to observe this activity on *Klebsiella pneumoniae* and *Escherichia coli* in the present study.

5 Conclusion

Lanosterol is a cyclic tetra triterpene compound that mainly integrates organic extracts of *Euphorbia aff. viridis (Klotzsch & Garcke) Boiss.* developed in the Andean region of Ecuador, and that could be used as chemical markers of the species studied. Organic extracts are generally safe compounds for their use in pharmacology and cosmetology, where their antimicrobial activity against *E. coli* and *K. pneumoniae* demonstrate their potential use as an antimicrobial agent and employment in ancestral medicine.

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