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Antibacterial activity of the *Croton draco* hidroalcoholic extract on bacteria of sanitary importance

Actividad antibacteriana del extracto hidroalcohólico *Crotón draco* sobre bacterias de importancia sanitaria

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ABSTRACT

The World Organization for Animal Health (OIE) mentions that the inappropriate use of antibiotics has led to the emergence of strains of resistant bacteria to the mechanisms of action of these drugs, due to the scarcity of effective therapies, the development of new treatment options is required for diseases that affect health, in this respect plant extracts or their pure compounds offer an alternative. The aim of the present investigation was to characterize and evaluate the in vitro antibacterial activity of the hydroalcoholic extract of *Croton draco* on bacteria of sanitary importance. The extract was obtained by hydroalcoholic maceration, a qualitative and chemical characterization of the extract was carried out, and the antibacterial activity was determined by the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). The qualitative characterization tests performed on the hydroalcoholic extract of *Croton draco* indicate the presence of phenolic compounds, terpenes, saponins and alkaloids, by gas chromatography the presence of thymol and carvacrol was determined in concentrations of 0.5340 mg/ml and 0.4206 mg/mL respectively. The bacteria showed different degrees of sensitivity to the hydroalcoholic extract, however, greater activity was determined against Gram positive bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus subtilis*.

Keywords: *Croton draco,* Hydroalcoholic extract, Antibacterial, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration.

RESUMEN

La Organización Mundial de Sanidad Animal (OIE) menciona que el uso inadecuado de antibióticos ha propiciado la aparición de cepas bacterias resistentes a los mecanismos de acción de estos fármacos, debido a la escasez de terapias efectivas se requiere del desarrollo de nuevas opciones de tratamiento para enfermedades que afectan la salud, al respecto los extractos de plantas o bien sus compuestos puros ofrecen una alternativa. El objetivo de la presente investigación fue caracterizar y evaluar la actividad antibacteriana *in vitro* del extracto hidroalcohólico de *Crotón draco* sobre bacterias de importancia sanitaria. El extracto se obtuvo mediante maceración hidroalcohólica, se realizó una caracterización cualitativa y

química del extracto, la actividad antibacteriana se determinó mediante la Concentración Mínima Inhibitoria (CMI) y la Concentración Mínima Bactericida (CMB). Los ensayos de caracterización cualitativa realizados al extracto hidroalcohólico de *Crotón draco* indican la presencia de compuestos fenólicos, terpenos, saponinas y alcaloides, por cromatografía de gases se determinó por primera vez la presencia de timol y carvacrol en concentraciones de 0.5340 mg/ml y 0.4206 mg/mL respectivamente. Las bacterias mostraron diferentes grados de sensibilidad, sin embargo, se determinó una mayor actividad frente a bacterias Gram positivas como *L. monocytogenes, S. aureus* y *B. subtillis.*
Palabras clave: *Crotón draco*, Extracto hidroalcohólico, Antibacteriano, Concentración Mínima Inhibitoria, Concentración Mínima Bactericida.

INTRODUCTION

The World Organization for Animal Health (OIE) mentions that the inappropriate use of antibiotics, has led to the emergence of bacteria strains resistant to the mechanisms of action of these drugs, this is an alarming situation for both human health and animal health (OIE, 2016), due to the lack of effective therapies. It requires the development of new treatment options for diseases that affect health, plant extracts or their pure compounds offer a potential alternative for the development of new antimicrobial treatments that can be used for the control of pathogenic microorganisms, due to the presence of metabolites or compounds that inhibit growth or cause their death (Lavor *et al*., 2014; Upadhyay *et al*., 2016).

In this regard Maxia *et al*., (2005) mention that there are ethno-veterinary practices by farmers and shepherds, based on knowledge transmitted from generation to generation. On the other hand Martínez and Jiménez-Escobar (2017), documented a total of 62 ethno-veterinary practices; highlighting the use of plants for the treatment of wounds, ocular and digestive conditions, practices associated with 44 plant species belonging to 30 botanical families; among which the genus *Croton* (*Croton lanatus*) stands out.
Dragon's blood, bloody or dragon's blood (La sangre de dragón, sangregado o sangre de dragon according its name in Spanish (*Croton draco*)), is one of the bushes used in traditional medicine by different cultures of the world; some of its ethno-medicinal uses are: hemostatic, antidiarrheal, antiulcer, antiviral, healing, antitumor, anti-inflammatory, antioxidant and antimicrobial, among others (Gupta *et al*., 2008).

In Mexico, this plant is attributed a lot of healing properties, such as those mentioned above, due to the wide range of secondary metabolites it has, such as: alkaloids, tannins, diterpenes and volatile oils (Salatino *et al*., 2007); however, studies are required to determine the concentrations at which it presents its pharmacological effect and the compounds to which the pharmacological properties can be attributed.

Studies conducted by Peres *et al*., (1997), show that extracts of some *Croton* species show inhibitory activity against *Staphylococcus aureus* and *Salmonella typhimuryum*. According to the aforementioned, the objective of the present investigation was to perform

a qualitative and chemical characterization of the hydroalcoholic extract of *Croton draco*, as well as determine its antibacterial activity (*in vitro*) on bacteria of sanitary importance.

MATERIAL AND METHODS

Obtaining the extract

Samples of plant material from the aerial part of *Croton draco* were collected during the summer (June-August) in the municipality of Huatusco (19 ° 08′56 ″ N 96 ° 57′58 ″ W) belonging to Veracruz state; for the identification of the plant, the herbarium of the National Autonomous University of Mexico was consulted, and the plant specimen was identified as *Croton draco* subsp. *draco* (IBUNAM: MEXU: 501697).

The plant material was dried at room temperature in the absence of light, 250 g of the dried material (crushed) was macerated in a liter of hydroalcoholic solution (70:30, water: methanol), for 48 hours at room temperature in the absence of light. The liquid extract from the maceration was filtered through filter paper and cotton; subsequently the liquid obtained was concentrated under reduced pressure in a rotary evaporator (BÜCHI™ R-210, Flawil, Germany), according to the methodology described by (Rivero-Pérez *et al*., 2016). The resulting extract was kept refrigerated until further evaluation.

Chemical characterization of the extract

To the hydroalcoholic extract of *Croton draco*, the qualitative chemical profile was performed according to the procedure described by Bañuelos-Valenzuela *et al*., (2018), in 10 ml Pyrex test tubes. The tests were as follows: KMnO4 test for unsaturations, FeCl3 test for phenolic oxydryls (vegetable tannins), Liebermann-Burchard test for sterols and triterpenes, Salkowski test for sterols and triterpenes, coumarins test, Baljet test for sesquiterpenlactones, H_2SO_4 test for flavonoids, Shinoda test for flavonoids, Dragendorff test for alkaloids, tannin test, floratanino test, steroid test, agitation test, sodium bicarbonate test and Salkowski test for saponins.

The chemical composition of the hydroalcoholic extract of *Croton draco* was determined according to the methodology described by Bañuelos-Valenzuela *et al*., (2018), using a gas chromatograph (CG; Agilent Tecnologies series 6890N manufactured in the USA), with a polar column DB_WAXetr, at 250 °C and 12.13 psi, with a flow of He 36.5 ml min^{-1} after injection. The conditions for the column were: initial temperature 50 ° C from zero to two min, increasing from 10 in 10 °C until reaching 250 °C, keeping the temperature constant for 5 min and then descending to 50 °C for two min with a flow of He of 1.6 ml min^{-1} at a pressure of 12.13 psi and an average speed of 25 cm s^{-1}, using an ionizing flame detector (FID), at a temperature of 210 °C, with a flow of H2 of 40 ml min^{-1} and an air flow of 450 ml min^{-1}. The standards (Sigma-Aldrich) were used in different concentrations (Table 1).

Table 1. Concentrations of standards used in gas chromatography for the chemical characterization of the hydroalcoholic extract of *Croton draco*

Standard	Timol (mg ml^{-1})	Carvacrol (mg ml^{-1})	Linalool (mg ml^{-1})	Terpinene (mg ml^{-1})	Limonene (mg ml^{-1})
1	10.373	8.284	7.744	7.154	8.496
2	5.186	4.142	3.872	3.577	4.248
3	2.593	2.071	1.936	1.789	2.124
4	1.297	1.035	0.968	0.894	1.062
5	0.648	0.518	0.484	0.447	0.531
6	0.324	0.259	0.242	0.224	0.265

Antimicrobial activity

The antibacterial activity was determined by the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) for the extract, following the CLSI specifications. (2012). Antimicrobial activity tests were carried out with the ATCC strains, *Staphylococcus aureus* ATCC ^{6538}, *Escherichia coli* ATCC ^{35218}, *Pseudomona aeruginosa* ATCC ^{9027}, *Salmonella typhi* ATCC ^{14028}, *Salmonella cholerasuis* ATCC ^{10708}, *Listeria monocytogenes* ATCC ^{19113} and *Bacillus subill* ATCC ^{6633} which were reactivated in Muller Hinton Agar (BD Bioxon), the purity of the strains was corroborated by Gram staining.

Once the morphology of the bacteria was confirmed, a colony was inoculated in nutrient broth (BD Bioxon), which was incubated under constant agitation (70 rpm) for 24 hours at 37 °C. After the incubation time, the inoculum was adjusted with nutrient broth to 0.5 of the McFarland turbidity pattern (Remel, R20421), which corresponds to 150 x 10^6 cell/ml. For the determination of the Minimum Inhibitory Concentration (MIC), the plate microdilution method was used; the concentrations evaluated were: 400, 200, 100, 50, 25, 12.50, 6.25, 3.12 mg/ml, for the strains to be evaluated. Each concentration was prepared with nutrient broth (BD Bioxon). The treatment was carried out in triplicate in a 96-well plate, 100 µl of each of the dilutions plus 10 µl of the bacterial suspension previously adjusted to 0.5 McFarland were placed. After inoculation, the plate was incubated at 37 °C for 24 hours at 70 rpm under constant stirring. The positive control was Kanamycin (AppliChem 4K10421) at concentrations of 64, 32, 16, 8.0, 4.0, 2.0, 1.0 and 0.5 µg/ml. Nutrient broth was used as a negative control.

To determine the end point of the MIC, a colorimetric method was used based on the use of tetrazolium salts, as described by Balouiri *et al.,* (2016). After the incubation time elapsed, 20 µl of a 0.04% (w / v) solution of p-iodonitrotetrazolium (Sigma-Aldrich I8377) was added to each well; it was incubated for 30 minutes at 37 ° C and the reading was performed, determining as the minimum inhibitory concentration; that to which the solution turns pink (Kaewpiboon *et al*., 2012; Mothana *et al*., 2009).

To determine the Minimum Bactericidal Concentration (MBC), after adding *p*-iodonitrotetrazolium, 5 µl of each well was inoculated into Mueller Hinton agar, then incubated at 37 °C for 24 hours. After the incubation time, the growth of the bacteria was verified to determine the minimum bactericidal concentration of the extract, fraction or

metabolite; considering as MBC, the concentration at which no bacterial growth was observed in the plaque.

Statistical analysis

The data obtained were normalized and analyzed by means of an analysis of variance and a comparison of means by Tukey, at a confidence level of 95 %; with the statistical package Minitab 18.

RESULTS

Chemical characterization of the hydroalcoholic extract of *Croton draco*

Qualitative characterization tests carried out on the hydroalcoholic extract of *Croton draco*, indicate the presence of phenolic compounds (coumarins, flavonoids and flavones), terpenes (sesquiterpenes), saponins and alkaloids.

The analysis in the gas chromatograph of the hydroalcoholic extract of *Croton draco* determined the presence of thymol and carvacrol in concentrations of 0.5340 mg/ml and 0.4206 mg/ml respectively; without determining the presence of linalool, terpinen and limonene.

Antibacterial activity

In the Minimum Inhibitory Concentration (MIC) of the *Croton draco* hydroalcoholic extract, significant statistical differences (P≤0.05) were determined between the bacteria evaluated, the MIC for *E. coli, S. typhi, S. cholerasuis, P. aeruginosa* was 100 mg/ml; without finding statistical differences, for L. monocytogenes of 50 mg/ml with significant differences with respect to the rest of the bacteria evaluated and of 25 mg/ml for *S. aureus, B. subtillis*; showing statistical differences with the aforementioned bacteria. On the other hand, the MIC for the bacteria evaluated showed significant statistical differences with respect to the MIC of the control with Kanamycin, as shown in Table 2.

Table 2. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the hydroalcoholic extract of *Croton draco*, on bacteria of sanitary importance

	Minimum inhibitory concentration		Minimum Bactericidal Concentration	
Bacteria evaluated	**EHCD (mg/mL)**	**Kanamycin (µg/mL)**	**EHCD (mg/mL)**	**Kanamycin (µg/mL)**
E. coli	100^{c}	4	200^{c}	8
S. typhi	100^{c}	4	200^{c}	8
S.cholerasuis	100^{c}	2	200^{c}	4
P. aeruginosa	100^{c}	16	200^{c}	32
L. monocytogenes	50^{b}	2	100^{b}	4
B. subtillis	25^{a}	0.5	50^{a}	1
S. aureus	25^{a}	2	50^{a}	4
P value	0.0001		0.0001	

EHCD; Croton draco hydroalcoholic extract, different literals ^{a,b,c} in the columns indicate significant statistical differences (*P*≤0.05)

In the Minimum Bactericidal Concentration (MBC) of the *Croton draco* hydroalcoholic extract, significant statistical differences (p≥0.05) were determined between the bacteria evaluated, the MBC for *E. coli, S. typhi, S. cholerasuis, P. aeruginosa* was 200 mg/ml;

without finding statistical differences between them, for *L. monocytogenes* of 100 mg/ml with significant differences with respect to the rest of the bacteria evaluated, and of 50 mg/ml for *S. aureus, B. subtillis*; showing statistical differences with the aforementioned bacteria. On the other hand, the MBC for the bacteria evaluated showed significant statistical differences (P≤0.05) with respect to the MBC of the positive control (Kanamycin), as shown in Table 2.

DISCUSSION

Currently, the emergence of bacteria resistant or multi-drug resistant to antibacterial drugs represents a serious public health problem worldwide; this problem requires constant vigilance, a situation that challenges the scientific community for the search for alternatives (Lai *et al*., 2017). Decreased efficacy and increased toxicity of synthetic antimicrobials aggravate the problem; so scientists are looking for natural compounds to get solutions. In this regard, traditional medicine based on the use of medicinal plants represents an alternative for the treatment of diseases caused by resistant or multirestant bacteria, both in humans and animals (Valle *et al*., 2015).

The results of the qualitative characterization of the hydroalcoholic extract of *Croton draco,* indicates the presence of phenolic compounds (coumarins, flavonoids and flavones), terpenes (sesquiterpenes), saponins and alkaloids; metabolites that have been reported in other plants with antibacterial activity, according to the literature: coumarins (Souzaa *et al*., 2005), flavonoids (Cushnie *et al*., 2005), sesquiterpenes (Barrero *et al*., 2005), saponins (Mandal *et al*., 2005) and alkaloids (Cushnie *et al*., 2014).
On the other hand, the chemical characterization by means of gas chromatography, allowed to determine the presence of thymol (0.5340 mg/ml) and carvacrol (0.4206 mg/ml); compounds with antibacterial activity according to Du *et al*., (2005).
Salatino *et al*., (2007) reported a qualitative and chemical characterization of different species of the *Croton* genus, which coincides with the characterization of the extract evaluated in the present experiment.

Peres *et al*., (1997) reported the antibacterial activity of the methanolic extract of Croton urucurana; determined a better activity of the extract against Gram positive bacteria (Staphylococcus aureus). Results that are consistent with those obtained in the present investigation, since the lowest concentrations of MIC and MBC were compared to the Gram positive strains of *L. monocytogenes* (50 mg / ml MIC, 100 mg/ml MBC), *B. subtillis* and *S. aureus* (25 mg/ml MIC, 50 mg/ml MBC). This can be explained because the outer membrane of Gram negative bacteria acts as a selective barrier that limits the entry of antibacterials (Cabrera *et al*., 2007).

Similar studies by Selowa *et al*., (2009), report the antibacterial activity of the methanol extracts of *Croton salvatycus, C. megalobotrys, C. steenkapianus*, against *E. coli*, *S.*

aureus and *P. aeruginosa*, being *C. steenkapianus* ; the only one inactive against *E. coli* and *S. aureus*. Case contrary to *Croton draco*, which maintains activity against these bacterial species; This difference may be due to the fact that the compounds and their concentrations change depending on the genus, species, phenological state, stress situations and geographical location of the plant; in addition to the technique used for the extraction and concentration of secondary metabolites (Hernández-Alvarado *et al*., 2018). Although in the present experiment the mechanism of action was not determined, the mechanism of action to which the antibacterial activity of coumarins, flavonoids, sesquiterpenes, saponins and alkaloids is associated, is due to its ability to affect the permeability of the cell membrane of Gram positive and Gram negative bacteria; Decrease the cytoplasmic pH and cause hyperpolarization of the cell membrane, according to what was published by Gonelimali *et al*., 2018.

CONCLUSION

The results indicate that in the hydroalcoholic extract of *Croton draco,* it contains metabolites with potential antibacterial activity; thus opening the possibility of being used as a phyto-pharmaceutical capable of acting against bacteria of sanitary importance; however, it is recommended in future studies to isolate and evaluate the compound (s) that confer said activity with collection strains and isolated field strains with resistance to different antibiotics.

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