Original Article

Analysis Of The Antimicrobial And Antioxidant Activity Of Essential Oils Of Royal Lemon (*Citrus Limonum*) Obtained By Two Extraction Methods In Ecuador

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Abstract

Citrus fruits are considered one of the major import crops worldwide, because they are grown in more than 140 countries. Being the lemon the most consumed citrus, the benefits of this citrus are widely known as they help strengthen the immune system in humans. Therefore, the objective in this research was to extract essential oil from the fruit bark and leaves of Royal lemon (*Citrus limonum*), by means of steam entrainment and fractional distillation, then the antimicrobial activity was evaluated by disc-plate diffusion, against the strains of *Escherichia coli*, *Salmonella*, *Listeria* and *Arcobacter*. The antioxidant activity of the essential oils obtained by the bleaching method with β-carotene "bleaching (BCB) test" was also analyzed. Finally, a Gas Chromatography analysis coupled to a Mass Spectrometer of the best treatment was carried out, to know which compounds are involved in the two bioactive principles (antimicrobial and antioxidant effect). Once these analyzes were carried out, it was determined that the oils obtained from the leaves of Royal Lemon have antimicrobial activity against *Listeria* and *Arcobacter*, for the antioxidant activity at a concentration of 50 mg/mL it presents a value of 48.82% and at a concentration of 100 mg/ mL presents 47.29% of sequestered peroxide, in short, it was evidenced that the best treatment was the essential oil of Royal lemon leaves by fractional distillation. After analysis by chromatography, the main compounds were identified as: Sabinene, D-Limonene, Linalool, Citronellal, Citronellol and 1R-α-Pinene, possibly being these the ones that presented the antimicrobial and antioxidant activity.

Keywords: Essential oil, royal lemon, steam entrainment, fractional distillation, antimicrobial, antioxidant.

INTRODUCTION

Citrus fruits are one of the major import crops worldwide, with lemon being the most consumed (**Sáenz et al., 2019**). Agro production in Ecuador is very varied, being citrus fruits such as lemon consumed by a large part of the population, the benefits of this citrus are widely known as they help strengthen the immune system and also fight cancer cells in humans. In Ecuador, of the varieties that currently exist, the Royal lemon (*Citrus limonum*) is widely cultivated in the Bolívar province, focusing its production on the subtropical zone such as: Las Naves, Echeandía and Caluma **(Solano, 2018).**

The Royal lemon or rough lemon is a crop of the citrus group that adapts very well to various types of soils, tolerating heavy and saline soils, it provides an exceptionally vigorous and productive tree from an early age with good quality fruit, it adapts well to light soils, with good drainage and soils with high pH **(Llamoca, 2017).**

The lemon is an acidic, yellowish fruit that is attributed great beneficial properties for health, one of which is its antioxidant action and high content of vitamin C. The lemon rind contains a large amount of soluble fiber, it has pectin that is located in the lower part of the rind. Phytonutrients are also found, the d-limonoid monoterpene is attributed benefits to combat some variants of cancer **(Talavera, 2018).** Lemon leaves contain phenolic compounds in large quantities, in addition to these they have phenolic acids, flavonoids, condensed tannins, so their consumption and use is of great contribution to health care and prevention of diseases that affect the nervous system, as well as gastrointestinal diseases. Due to its properties and active ingredients, it is used on a large scale in industries related to cosmetology, perfumery, pharmaceuticals and food. **(Luna et al., 2019).**

The essential oil is applied as a microbial inhibitor due to its components to eliminate pathogens such as *E. coli*, *Salmonella typhmurium*, *S. aureus*, *Campylobacter*, *Enterococcus fecalis*, *Listeria monocytogenes*, *Arcobacter* **(Lin et al., 2016).** According to **Morales (2020),** he considers that antimicrobials can be antibacterial, antiviral or antifungal, classifying them as bactericidal or bacteriostatic. Therefore, the object of study was to analyze the antimicrobial and antioxidant activity of royal lemon essential oil obtained by two extraction methods, steam distillation and fractional distillation.

MATERIALS AND METHODS

Research location

The research was carried out in two phases, the first corresponds to the obtaining of raw material in the Caluma canton, Bolívar province, the second was carried out in the facilities of the General Laboratory of Laguacoto I of the State University of Bolívar.

Study factors and experimental design

To carry out the present investigation, two study factors were considered: Factor A, raw material (peel and leaves) and factor B, extraction methods (distillation by steam dragging and fractional distillation).

A Completely Random Design (D.C.A) was applied because all the conditions were controlled, where factor A represents the raw material, factor B methods for the extraction of essential oil; obeying a factorial arrangement AxB with 2 repetitions obtaining 8 treatments (2x2x2).

Collection of raw material

The experimental material such as fruits and leaves of Royal lemon was collected in the town of Embarcadero belonging to the Caluma canton, province of Bolívar-Ecuador. Selection (Lemons with a diameter of 8.4 to 8.8 cm and leaves with a length of 7 to 14.5 cm and a width of 4 to 8 cm were selected); Washing (to remove foreign agents and other types of microorganisms); dried and chopped**.**

Obtaining essential oils

Extraction by steam dragging method

For the extraction of essential oil from the rind and leaves of Royal lemon, a steam dragging equipment was used, which consists of the following parts: Heating mantle, a 1,000 mL flask, distillation head adapted with thermometer, ball refrigerant, distillation elbow, separatory funnel and universal supports are used to support the structures.

Extraction by fractional distillation method

To obtain the essential oil from the rind and leaves of Royal lemon, a Fractional distillation equipment was used, which consists of the following parts: Heating mantle, a 1,000 mL flask, fractionation column, distillation head adapted with thermometer, ball condenser, distillation elbow, separatory funnel and a universal support is used to support all the structures.

Essential Oil Yield

Once the essential oil was obtained from both the bark and the leaves of the Royal lemon by the two extraction methods, the yield calculation was carried out by applying the following equation described by **Melo et al. (2020).**

$$P = (M_1 \backslash M_2) * 100$$

Where: P= Yield; M_1= It is the final mass of the essential oil; M_2= The initial mass of the foliage.

To determine the best performance of the extractions, the Tukey test was applied.

Determination of antimicrobial activity

We worked with previously characterized strains of *E. coli, Salmonella, Listeria* and *Arcobacter*, belonging to the Bank of Microorganisms of the Molecular Biology Laboratory belonging to the State University of Bolívar.

Bacterial reactivation

We worked with 12 isolates of *E. coli*, *Salmonella*, *Listeria* and *Arcobacter*, three of each. The process was carried out by pouring 0.5 mL of culture (cryovial), into petri dishes of nutrient agar (7145A, Acumedia, USA) for *E. coli*, XLD "Xylose Lysine Desoxycholate" agar (7166A, Acumedia, USA) for *Salmonella*, Agar Aloa Listeria agar base + Blood Agar Base Infusion Agar (TM1443, TM media, India + 211037, BD, France), for *Listeria* and Arcobacter broth agar + Bacto agar + sheep blood (CM0965, Oxoid, England + 214010, BD, France) for *Arcobacter*, finally, the strains were incubated at 37 °C for 24 or 48 hours as appropriate, aerobiosis in the case of *E. coli*, *Salmonella*, *Listeria* strains and under microerophilic conditions (10 % CO_2, 5% O_2, and 85% N_2) for the *Arcobacter* strain.

Antimicrobial activity analysis of essential oils using the Kirby Bauer method (disk-plate diffusion).

A suspension was made with 10% saline solution to a turbidity of 0.5 on the McFarland scale, to reduce the bacterial load of the reanimated strains.

With a sterile swab, seeding was carried out on Müller Hinton Agar plates (225250, BD, France) in a uniform manner, then the disks were placed on the surface of the agar that had previously been submerged in the essential oil (12 blank disks). PQ/50 of 6 mm for each oil). Likewise, Ciprofloxacin and Streptomycin discs were tested as controls. Subsequently, the plates were incubated at 37 ºC under controlled conditions as appropriate. Finally, the diameters of the inhibition halos of the discs were measured, and the results were analyzed according to the criteria indicated by the Institute of Standards and Clinical Laboratory **(CLSI), and Ponce, (2007).**

Determination of antioxidant capacity

The antioxidant capacity was determined by the β-carotene bleaching method "bleaching (BCB) test", described by **Kulisic et al. (2004),** where the H_2O_2 solution (40 mM) was prepared in a monobasic sodium hydroxide solution potassium phosphate (50 mmol; pH 7.4). The concentration of H_2O_2 was determined by absorption at 230 nm in a GENESYS™ 10S Visible Spectrophotometer (Thermo Electron Corporation, 335902, USA). Subsequently, the oils were added individually at concentrations of 50 µg/mL and 100 µg/mL in H_2O_2, the absorbance was determined at 230 nm after 10 min against blank solutions containing phosphate buffer with H_2O_2. Subsequently, the % scavenging of hydrogen peroxide is calculated with the following formula:

Antioxidant capacity (% of H_2O_2 sequestered) = ((Ai-At)/Ai) x 100
Where: Ai= Absorbance of the reference standard; At= Absorbance of the simple

Determination by Gas Chromatography (GC-MSD).

In order to know the components that intervene in the antioxidant and antimicrobial effects, a gas chromatography coupled to mass spectrometry was carried out. In the best treatment (essential oil of Royal lemon leaves obtained by fractional distillation). For sample preparation, 80 µL of sample and 160 µL of hexane were placed in an insert, which was shaken in a vortex for 2 min. For the identification of compounds, it was carried out in a gas phase chromatograph combined with a mass detector. An HP-5MS column (30 m × 0.25 mm × µm) and helium 5.0 as carrier gas were used. Helium was used with a flow rate of 0.8 mL/minute, 0.1 µL of sample was injected in splitless mode, the injection T was 250 °C, the oven T was: 60 °C for 1 minute increased at 126 °C at a rate of 6 °C / minute, then the temperature rises to 165 ° C at a rate of 1.5 °C / minute, finally it increased to 290 °C by a ramp of 8 °C / minute and is held for 6 min at the final temperature. The T of the mass detector was 260 °C in full scan mode from 40-450 AMU. Compounds were identified using the NIST 14.L library.

RESULTS AND DISCUSSION

Yield of the essential oil obtained by each extraction method.

Steam stripping

Table 1. Yield of essential oil by steam drag method.

Variables	Peel	Leaf
Sample weight (g)	700	700
Essential oil weight (g)	4.3	1.7
% Yield	0.61	0.24

Table 1 shows the percentage of essential oil yield extracted by steam dragging with two repetitions, both from the peel and from the Royal lemon leaves. It is evident that the oil from the leaves has a lower yield compared to the yield of the oil from the peel.

This can be attributed to the fact that the leaves, being thinner, store a low amount of essential oil. The yield of essential oil from the peel in our research is higher than those reported by **Córdova & Velásquez (2021**), in which they report a yield of 0.5% of essential oil from lemon peel, likewise, **Laso & Jimeno (2018),** in their research they obtained a yield of 0.31% obtained from lemon, a value lower than that obtained in the present investigation. While the yield in essential oil of leaves in our research is lower than that reported by **Ortega (2018)**, where he obtains a yield of 0.95% in leaves of Persian lemon (*Citrus latifolia* Tanaka), in the same way **Medrano (2019),** shows an essential oil yield of 0.23% in lemon (*Citrus aurantifolia*), a value close to that of our work. In this sense, these values vary significantly either by the lemon variety or by the cultivation area.

Fractional distillation method

Table 2. Yield of essential oil by fractional distillation method

Variables	Peel	Leaf
Sample weight (g)	700	700
Essential oil weight (g)	3.8	1.3
% Yield	0.54	0.18

Table 2 shows the results of the percentage of essential oil yield extracted by fractional distillation with two repetitions, both of the peel and of the Royal lemon leaves. It is evidenced that the oil from the bark has a higher yield compared to the yield of the oil from the leaves. This can be attributed to the fact that the casacara has eyes that store essential oil in greater quantity, unlike the leaves, which are thin and smooth, and contain essential oils in low quantities. Regarding the extraction of essential oil from lemon, both from the peel and from the leaves by the fractional distillation method, no bibliographical references were found, consequently, our work is the first to be carried out.

Determination of the antimicrobial and antioxidant activity of the essential oil

Analysis of antimicrobial activity of oils against *Escherichia coli*.

Table 3. Antimicrobial capacity of oils obtained against to *Escherichia coli*.

Strains of *E. coli*	Treatments				Control antibiotics	
	T1	T2	T3	T4	Ciprofloxacin	Streptomycin
Cer 60	1	1	3.5	1	25	29
Cer 62	1	1	1	8.5	27	31
Cer 61	1	1	9	7	25	29

Table 3 shows the results of the inhibition halos of the antimicrobial activity of the essential oil of Royal lemon both from the peel and from the leaves by the two extraction methods against the *E. coli* strains. In the study by **Vignola et al, (2020),** they revealed that in the extract of lemon (*Citrus lemon*) obtained by steam dragging, for the antimicrobial activity against the bacteria *E. coli*, the authosrs obtained an inhibition halo of 9.66 mm in diameter, thus calling it moderate antimicrobial activity, this value agrees with the values of our research in T3 and T4 against strains Cer 61 and Cer 62 respectively. While **Espinel (2020**), reports an inhibition halo of 1.5 mm in diameter in the extract of lemon (*C. lantifolia*) obtained by steam dragging against the bacteria *E. coli*, a value equal to the treatments T1 and T2 of our study. On the other hand, **Montero (2009),** in his research, mentions that there is no antimicrobial activity in lemon extract (*C. lantifolia*), extracted by hydrodistillation against *E. coli* bacteria. The data obtained in our study differs significantly from that of other researchers, possibly due to the variety of lemons and extraction methods.

Analysis of antimicrobial activity of oils against *Salmonella* sp.

Table 4. Antimicrobial capacity of oils obtained against *Salmonella* sp.

Strains of *Salmonella*	Treatmets				Control antibiotics	
	T1	T2	T3	T4	Ciprofloxacin	Streptomycin
Suelo 58	1	1	3	5	21.5	18
S7	1	1	6	6,5	24	16
SM3	1	2	8	7	23.75	19

Table 4 shows the results of the antimicrobial activity of the essential oil of Royal lemon, as well as the peel and leaves by the two extraction methods, against the *Salmonella* strains, which has an antimicrobial effect. It is important to note that according to **Ponce, (2008),** for there to be an inhibitory effect of oils and natural extracts, the size of the halo must be a minimum of 8 mm, so that it can be considered that the oils do have an inhibitory effect. clear against the *Salmonella* genus. In the work carried out by **Jiménez et al, (2013**), in the sweet lime extract obtained by maceration, they revealed an inhibition halo of 30 mm in diameter against *Salmonella*, a value very different from that of our research. , in another study carried out by **Blas & Fernandez (2021**), obtained a result of a 6 mm diameter halo of inhibition of citrus sinensis essential oil on *Salmonella* bacteria, data identical to those of our work in T3 and T4 in the S7 strain, that is to say that it does not present antimicrobial activity unlike T3 and T4 in the SM3 strain exceeds 6 mm in diameter, therefore these treatments do present antimicrobial activity. **Torres (2018),** in his research carried out on the essential oil of orange, reveals an inhibition halo of 16 mm in diameter, a value identical to the control antibiotic ciprofloxacin in the S7 strain and higher than the inhibition halos of the essential oil of orange. our investigation. Regarding the antimicrobial activity of lemon essential oil by the two extraction methods, no reports were found in the literature in such a way that comparisons with citrus fruits were made, therefore, our research work is the first to be carried out.

Analysis of antimicrobial activity of oils against *Listeria sp*.

Table 5. Antimicrobial capacity of oils obtained against *Listeria sp.*

Strains of *Listeria*	Treatments				Control antibiotics	
	T1	T2	T3	T4	Ciprofloxacin	Streptomycin
L.28	1	1	34.5	31	38.75	30.25
PTI	2,5	5	39.5	40	33.75	27.75
L.36	1	1	25	25	36.5	32.75

Table 5 shows the antimicrobial action results of the essential oils of both the peel and the leaf by the two extraction methods against *Listeria* strains, which exceed the range of the inhibition halos of the control antibiotic of both ciprofloxacin and streptomycin, thus demonstrating that there is antimicrobial activity in these treatments, while treatments T1 and T2 have low antimicrobial activity since the inhibition halos is much lower than those of the control antibiotics used. In addition, the halo diameter values are above the 8 mm recommended by **Ponce (2008**). **Tinoco (2020)**, carried out a study showing an inhibition halo of 8.7 mm in diameter in lemon extract (citrus latifolia), a value different from that of our research, **Torres (2018),** in his research presents a value of 12 mm diameter of inhibition halos in orange essential oil on *Listeria* bacteria, a value that is lower than the T3 and T4 of our work, presenting low antimicrobial activity, finally **Córdova et al, (2020),** demonstrated a halo of inhibition of 18.2 mm in diameter in the orange essential oil obtained by the microwave extraction method where it is reported that an inhibition scale ≥ 20 mm is strongly inhibitory; it is moderately/mildly inhibitory when it has an area <20-12 mm; and <12 mm is not inhibitory, therefore, the values of the present investigation in T3 and T4 present strong antimicrobial activity with values of 25 to 40 mm in diameter.

Analysis of antimicrobial activity of oils against *Arcobacter* sp.

Table 6. Antimicrobial capacity of oils obtained against *Arcobacter* sp.

Strains of *Arcobacter*	**Treatments**				**Control Antibiotics**	
	T1	**T2**	**T3**	**T4**	**Ciprofloxacin**	**Streptomycin**
Q3NC2	1	1	27.5	15.5	24.75	15.5
Q1NC1	30	5	32	40	44	33
Q18BC1	34	19.5	46	46	41.75	28

Table 6 shows the results of the antimicrobial activity of the essential oil obtained from Royal lemon both from the peel and from the leaves by the two extraction methods, in which there is antimicrobial activity against the *Arcobacter* strains. **Ponce et al, (2008)**, considers that an acceptable degree of susceptibility of a microorganism to agents of plant origin must be a minimum of 8 mm. According to **Vidal (2017)** and **Bayas-Chacha et al. (2022),** in their research work, they determine the breakpoints of ciprofloxacin on *Arcobacter*, where he mentions that it is resistant when the inhibition halo is ≤ 20 mm in diameter and susceptible when the inhibition halo is ≤ 20 mm in diameter is ≥ 24 mm in diameter, therefore, the values of our research have antimicrobial activity in the essential oils of Royal lemon against *Arcobacter* since their values are between 27.5 to 46 mm in diameter, **Bayas-Morejón et al. (2020**), reveals an average value of 31.9 mm in diameter of the inhibition halo of the antibiotic streptomycin against *Arcobacter,* a value similar to that of our research except for the Q3NC2 strain that has a value of 15.5 mm in diameter, according to the recommendations given by the **CLSI (2017),** for antimicrobial susceptibility tests by the disk-plate diffusion method using ciprofloxacin against *Arcobacter*, reports that it is resistant when the inhibition halo is ≤ 12 mm in diameter and susceptible when it is ≥ 24 mm in diameter, that is, the values obtained in our work show antimicrobial activity. Regarding the antimicrobial activity of the essential oil of Royal lemon against the *Arcobacter* bacteria, no information was found in the literature, therefore, the present investigation is the first to be carried out in such a way that the interpretation was made by comparing the halos inhibition of control antibiotics.

Analysis of antioxidant activity

Antioxidant analysis by the ß-carotene bleaching method "bleaching (BCB) test".

Table 7. Antioxidant capacity (% sequestered peroxide) of the oils obtained.

Treatments	**(% sequestered peroxide Concentration (mg/mL)**	
	50	**100**
T1	25.58	16.31
T2	26.08	47.29
T3	9.19	30.00
T4	48.82	36.04

In table 7, The results of the antioxidant activity of the essential oil of Royal lemon are shown, both from the bark and from the leaves by the two extraction methods, these values being the most representative in comparison to the research carried out by **Del Toro et al. (2015)**, where the authors disclose a result of 30.39 EC50 (mg/mL) in lemon essential oil by the ß-carotene discoloration inhibition method, while **Miranda (2017),** by the same method, obtained a value of 4.03 EC50 (mg/mL) but in tangerine essential oil of the basol variety, the amounts reported by the authors differ significantly from those of our work, being able to attribute to the *citrus* variety, to the method of obtaining essential oil , the state of maturation, the place where it was harvested and the climatic conditions. On the other hand, **Álvarez et al. (2015),** carried out an investigation of the antioxidant activity by the DPPH method where they obtained a result of 1.05 IC50 mg/mL in *citrus latifolia* lemon essential oil extracted by maceration.

Identification of volatile compounds by gas chromatography of the essential oil obtained.

With the analysis of the volatile compounds, antimicrobial and antioxidant effects were identified, among these we have Sabinene with an area of 14.21%, followed by D-Limonene with an area of 12.09%, Linalool with an area of 7.68%, Citronellol with an area of 6.35% and Citronellal with an area of 5.98%, and finally we have 1R-α-Pinene with an area of 4.18%. **Ponce (2017),** in the essential oil of grapefruit peel (*Citrus paradisi* L), to which he attributes antimicrobial action properties; Linalool was also found in our research, the same one that was identified by **Argote et al, (2017),** in the essential oil of mandarin, this compound is widely studied for its antibacterial and antifungal properties. **Becerra & Castro (2019)**, in their research, identified the compounds Citronellal, Geraniol, Citronellol, and β-Citral, in the Limonaria and Citronella plants, where they mention that these compounds have antimicrobial and antioxidant potential, also identified by **Ponce (2017).** In the essential oil of grapefruit peel (*Citrus paradisi* L), to which it attributes antimicrobial action properties. **Mahdian et al, (2017),** identified the Caryophyllene compound in the essential oil of Echinophora platyloba, this compound was also found in the present work with an area of 2.00% and a retention time of 17.586 min which acts as antimicrobial and antioxidant.

CONCLUSION.

In the analysis of the antimicrobial activity of the oils obtained against pathogenic bacteria such as: *E coli*, *Salmonella*, *Listeria* and *Arcobacter*, it was shown that there is greater activity in the essential oil of Royal lemon leaves (*Citrus limonum*) by fractional distillation against *Listeria* with an inhibition halo of 40 mm in diameter, in the same way there is also greater antimicrobial activity in the essential oil of Royal lemon leaves by steam entrainment and fractional distillation against the *Arcobacter* bacteria, the same one that has an inhibition halo of 46 mm in diameter, this given that the inhibition halos of the essential oil are similar to the inhibition halos of the control antibiotic used, while for *E coli* and *Salmonella* bacteria there is low antimicrobial activity.

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