Vol. 2, No. 2 (2023) 01-09 http://sniqba.com.mx/revsniqba-2023-02-01

*Revista SNIQBA*

# EXTRACTION OF BIOACTIVE COMPOUNDS FROM *ROSMARINUS OFFICINALIS* AND *THYMUS VULGARIS*: COMPARISON OF CONVENTIONAL *VS*. MICROWAVE METHOD

# EXTRACCIÓN DE COMPUESTOS BIOACTIVOS DE *ROSMARINUS OFFICINALIS* Y *THYMUS VULGARIS*: COMPARACIÓN DEL MÉTODO CONVENCIONAL CONTRA MICROONDAS

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**Received:** August 24, 2023 ***Revised:*** *September 27, 2023* ***Accepted:*** *October 29, 2023*

## Abstract

*Rosmarinus officinalis* L. (rosemary) and *Thymus vulgaris* L. (thyme) are Mediterranean plants that have demonstrated diverse properties (antimicrobial, antioxidant, antiseptic, anti-inflammatory, etc.), thanks to which the extracts of these plants are used for different applications. However, to obtain a good extraction yield, as well as bioactive compounds, it is necessary to determine the extraction methodology. The extraction methods most frequently used are conventional (maceration, Soxhlet distillation, etc.). However, non-conventional methods, such as microwaves or ultrasound, have been utilized due to being considered green energies since the use of these methods can reduce extraction time and improve yields. Therefore, in this research, ethanolic extracts of *Rosmarinus officinalis* and *Thymus vulgaris* were obtained using two different extraction techniques, one conventional (solid-liquid) and the other assisted with microwaves. The bioactive compounds present in the extracts were rosmarinic, carnosic, caffeic acids, rosmanol, quercetin, and luteolin. These were identified by FTIR-(ATR) and HPLC. The extracts demonstrated heat resistance above 200 °C. The extracts MRE, CRE, and CTE showed a better inhibition to *E. coli*, whereas the ETC extract showed a better inhibition of *S. aureus*. Due to the results obtained, both extraction methods are reliable for obtaining bioactive compounds from plants of the Lamiaceae family, compounds that could be used in food, cosmetics and/or pharmaceuticals.

***Keywords****: Rosemary, Thyme, Extracts, Bioassays*

## Resumen

*Rosmarinus officinalis* L. (romero) y *Thymus vulgaris* L. (tomillo) son plantas mediterráneas las cuales han demostrado poseer diversas propiedades (antimicrobianas, antioxidantes, antisépticas, antiinflamatorias, etc.), gracias a las cuales, los extractos de estas plantas han sido utilizados en diferentes aplicaciones. Sin embargo, para lograr obtener un buen rendimiento de extracción, así como, compuestos bioactivos, es necesario determinar

bien la metodología de extracción. Los métodos de extracción más comúnmente empleados son los convencionales (maceración, destilación Soxhlet, etc), por otro lado, se han utilizado métodos no convencionales como lo es el microondas o el ultrasonido, gracias a que se consideran energías verdes, ya que con el uso de estos se puede reducir el tiempo de extracción y mejorar los rendimientos. Por lo que, en esta investigación se obtuvieron extractos etanólicos de *Rosmarinus officinalis* y *Thymus vulgaris*, mediante dos técnicas de extracción diferentes, una convencional (sólido-líquido) y otra asistida con microondas. Los compuestos bioactivos presentes en los extractos fueron ácidos rosmarínico, carnósico, cafeico, rosmanol, quercetina, luteoil, etc. Los cuales fueron identificados mediante FTIR-(ATR) y HPLC. Todos los extractos demostraron una resistencia térmica por encima de los 200 °C. Los extractos de MRE, CRE y CTE obtuvieron una mejor inhibición frente *E. coli*, mientras que para *S. aureus* fue el extracto ETC. Debido a los resultados obtenidos, ambos métodos de extracción son confiables para la obtención de compuestos bioactivos a partir de plantas de la familia Lamiaceae, para el área de alimentos, cosmética y/o farmacéutica.

**Palabras clave**: Romero, Tomillo, Extractos, Bioensayo

## Introduction

Mediterranean plants have attracted great interest from different researchers due to the beneficial characteristics they have been shown to possess, attributed to their high content of bioactive compounds, the main ones being polyphenols and carotenoids (Munekata et al., 2020), among them are properties such as antiviral (Sanna et al., 2015), antimicrobial, antioxidants (Alirezalu et al., 2020), anti-inflammatory (Conforti et al., 2008), etc.

The Lamiaceae family are Mediterranean plants that are widely used for culinary purposes, as spices, conservators, and antioxidants, as well as in medicine, like antiseptic, due to the wide variety of phytochemicals they contain (Calinescu et al., 2017; Milevskaya et al., 2017). Specifically, *Rosmarinus officinalis* L. and *Thymus vulgaris* L., commonly called rosemary and thyme, respectively, are plants native to the Mediterranean basin and are widely used as food flavoring and conservatives (Calinescu et al., 2017). Moreover, they are well known for their antibacterial, antifungal, antioxidant (Ojeda-Sana et al., 2013; Vallverdú-Queralt et al., 2014), anti-inflammatory, antiviral, antimutagenic, antiseptic, insecticidal properties (Calinescu et al., 2017).

Extracts of *Rosmarinus officinalis* and *Thymus vulgaris* are commonly rich in bioactive compounds. Rosmarinus officinalis are mainly rosmarinic acid and carnosic acid (Jacotet-Navarro et al., 2016), whereas *Thymus vulgaris* typically contains rosmarinic acid, caffeic acid, hydroxybenzoic acid (Vallverdú-Queralt et al., 2014). However, the constitution of bioactive compounds in the extract depends on different factors, such as extraction method, extracting solvent, temperature, pressure, time, part of the plant, etc. (Andrade et al., 2018). Therefore, the extraction technique is the key to obtaining an efficient process with high yields. The most common methods used are conventional (maceration, Soxhlet extraction, solid-liquid extraction, hydrodistillation, etc.). Nevertheless, new extraction techniques, considered more eco-friendly, have been developed and used in recent years, including microwave-assisted and ultrasound-assisted extraction (Elyemni et al., 2019; Moreira et al., 2017).

The bioactive compounds obtained from the extracts of *Rosmarinus officinalis* and *Thymus vulgaris*, regardless of the extraction method, can be used in different areas, such as in food for the development of packaging materials, storage(Stahl-Biskup & Venskutonis, 2012), cosmetics as skin conditioning agents or as fragrance ingredients (Fiume et al., 2018), in pharmaceuticals as antiseptics, anti-inflammatories (Alu'datt et al., 2018), etc.

Therefore, in this research, the ethanol extraction of bioactive compounds from *Rosmarinus officinalis* and *Thymus vulgaris*. Through two different methodologies, one conventional (solid-liquid extraction) and the other microwave-assisted, to compare both extraction methods for their potential use in food, cosmetics and/or pharmaceutical applications.

## Experimental section

The leaves and stems of *Rosmarinus officinalis* and *Thymus vulgaris* were used to obtain the bioactive compounds. These were dried at room temperature and subsequently ground. Then, 100 g of the plant was placed in a reactor, and 1 L of absolute ethanol was added. The conditions for the conventional method (MC) were 70 °C for 30 min with magnetic

stirring of 600 rpm, while for the microwave method (MM) 70 °C for 30 min at 800 W. They were then filtered and rota evaporated to obtain the bioactive compounds from each plant (*Rosmarinus officinalis* extract by microwave (MRE), *Rosmarinus officinalis* by conventional (CRE), *Thymus vulgaris* by microwave (MTE) and *Thymus vulgaris* by conventional (CTE)).

The yield percentage, as well as the extraction conditions, are described in Table 1.

Table 1. Conditions and extraction yields by MC and MM.

| Plant | Microwave | Yield (%) | Conventional | Yield (%) |
|---|---|---|---|---|
| *Rosmarinus officinalis* | 30 min, 70 °C, 800 W | 8 | 30 min, 70 °C, 600 rpm | 15 |
| *Thymus vulgaris* | 30 min, 70 °C, 800 W | 5 | 30 min, 70 °C, 600 rpm | 8 |

The extracts obtained were analyzed by infrared spectroscopy (IR Spectrum spectrophotometer, GX-Perkin-Elmer employing the Attenuated Total Reflection (ATR) technique, using a diamond tip attachment, FTIR-(ATR)), high-performance liquid chromatography (Varian Prostar, with diode array detector (280 nm). 1.8 mL of sample was filtered (0.45 µm membranes). Separation of the compounds was performed on a Grace Denali c-18 column at 30 ° C. Mobile phase A was methanol (wash), B acetonitrile, and 3% acetic acid C, flow rate 1 mL/min, and injection volume 10 µL. Mass analysis was performed using a Varian 500-MS ion trap, electrospray ionization (ESI), capillary voltage 90 V, negative mode ([M-H] - m/z), and mass acquisition range 100-2000 m/z, HPLC-MS). For this thermogravimetric analysis (TGA), a TA instruments Q500 thermal analyzer was used, with a heating rate of 10 °C/min and a temperature range of 30-800 °C. Bioassays of antimicrobial activity were carried out against *E. coli* and *S. aureus*, for which dilutions of the cultures of the pathogenic bacteria were made up to $1x10^{-3}$. Then, 300 µL of the last dilution was placed with a 1000 µL automatic pipette (SCILOGEX brand) on the Petri dish with nutrient agar, spread with a sterile isopod, and finally, four sterile filter paper disks were placed on the Petri dish, and 7 µL of the extract were placed on each one, each extract was evaluated in duplicate with the different bacteria to obtain a total of eight replicates, these were left incubating for 24 h and after this time was observed to verify the presence or absence of inhibition, determined by a halo of inhibition around the disc, compared with the control.

## Results

FTIR-(ATR) analysis was carried out to know the functional groups present in the samples. Figure 1 shows the IR spectra of *Rosmarinus officinalis* extracts, where influential bands with wave numbers at 3351 $cm^{-1}$ vibration of -OH bond, 2919 $cm^{-1}$ stretching of $-CH_3$, 2850 $cm^{-1}$ stretching of $-CH_2$, 1685 $cm^{-1}$ vibration of C=O, 1453 $cm^{-1}$ tension of C=C bond, 1274 cm-1 vibration of O-H and at 1031 $cm^{-1}$ stretching of C-O are observed. This coincides with what was reported by Mohammed et al. in 2022; in their research, they performed extracts of rosemary, using absolute ethanol as solvent, and the FTIR analysis presented main bands at 3299 $cm^{-1}$ attributed to the presence of alcohol, at 2922, 2832, 2993, 2981 $cm^{-1}$ coming from the $-CH_3$ and $-CH_2$ groups present in chlorophyll groups and 1152, 11029 and 1057 $cm^{-1}$ the vibration of the C-O bond due to the presence of acids in the sample (Mohammed et al., 2023). Also, Hameed, et al. and Piñeros et al. report that these bands are due to the presence of rosmarinic and carnosic acid, which are the most frequently extracted in rosemary ethanolic extracts (Hameed et al., 2015; Piñeros Hernández et al., 2017).

The infrared spectra for Thymus vulgaris are presented in Figure 2. In these, important bands are observed at wavenumbers 3337 $cm^{-1}$ due to the vibration of the -OH bond, 2924 and 2850 $cm^{-1}$ due to the stretching of the $-CH_3$ and $-CH_2$ bonds, respectively; 1688 $cm^{-1}$ the tension of the C=O bond, 1447 $cm^{-1}$ the stretching of C=C, 1268 cm-1 the vibration of the C-C bond, 1030 $cm^{-1}$ stretching of the C-O bond and 806 $cm^{-1}$. Ardjoum and collaborators, in 2021, performed ethanolic extractions of thyme, where they reported bands with wave numbers at 3407, 2962, 2876, 1614, 1422, and 811 $cm^{-1}$. They mention that the bands between 1614 and 1422 $cm^{-1}$ are assigned to the phenolic ring present in the thymol. In addition, the peak at 811 $cm^{-1}$ is due to vibrations of the C-H bond coming from an out-of plane aromatic ring (Ardjoum et al., 2021).

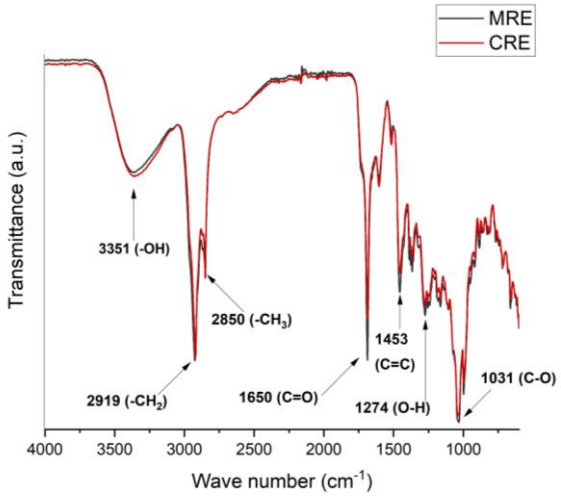


Figure 1. FTIR-(ATR) spectra of *Rosmarinus officinalis* extracts.

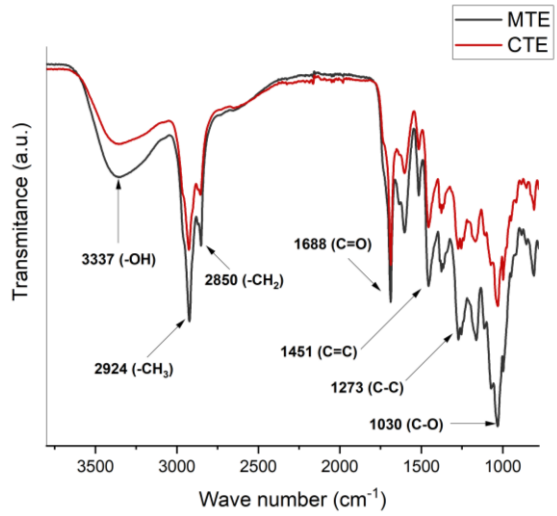


Figure 2. FTIR-(ATR) spectra of *Thymus vulgaris* extracts.

Thus, according to the results obtained, it is possible to elucidate that the extracts contain phenols, which are considered to be one of the main components responsible for the antimicrobial activity of the extract (Cervilla et al., 2012; Nasrollahzadeh et al., 2016).

HPLC-MS analysis was then performed to determine the family and compounds present

in each of the extracts. Table 2 and 3 describes each compound and family found for each extract. The compounds identified by HPLC for both extracts using the different extraction methodologies were mainly rosmarinic acid, rosmanol, caffeic acid, coumarin, quercetin, luteolin, carnosic acid, and hydroxybenzoic acid. These results are similar to what is reported in the literature, with the main bioactive compounds present in *Rosmarinus officinalis* being rosmarinic acid, rosmanol, carnosol, caffeic acid, luteolin, feruloic acid, quercetin, coumarinic acid, catechol, hydroxybenzoic acid, vanillic acid, cinnamic acid (Ezzat et al., 2016; Hisham et al., 2022; Kanakidi et al., 2022; Luță et al., 2023). And for *Thymus vulgaris* extract the most frequently found bioactive compounds are rosmarinic acid, quercetin, coumarinic acid, caffeic acid, rutin, quercetin, gallic acid, ferullic acid, catechol, luteolin, hydroxybenzoic acid, among others (Luță et al., 2023; Shahar et al., 2023).

The thermograms of the extracts of *Rosmarinus officinalis* and *Thymus vulgaris* are presented in Figure 3. The thermal stability of the extracts was determined by this analysis (TGA). In the case of the *Rosmarinus officinalis* extract, a degradation temperature of approximately 224 °C was obtained. This is similar to that reported by Piñeros et al. who obtained an ethanolic extract of rosemary by a conventional method, which presented degradation above 200 °C (Piñeros Hernandez et al., 2017). Moreover, *Thymus vulgaris* extract presented a degradation temperature of 204 °C. In 2020, and collaborators, reported that the thermal stability of an extract of *Thymus vulgaris* is above 190 °C, so it agrees with the obtained result (Radünz et al., 2020).

Therefore, we can elucidate that the degradation temperature of the extracts of Rosmarinus officinalis and Thymus vulgaris, obtained by conventional and microwave-assisted techniques, is above 200 °C. Hence, it is determined that the extracts do not suffer any chemical decomposition during the extraction and recovery process.

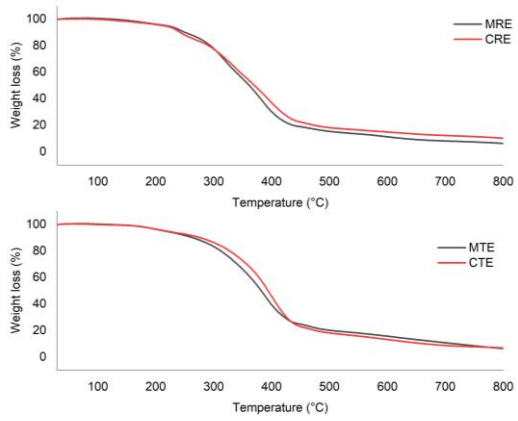


Figure 3. Thermograms of the extract of *Rosmarinus officinalis* and *Thymus vulgaris.*

Table 2. Compounds identified by HPLC of *Rosmarinus officinalis* by conventional and microwave-assisted extraction.

| Extract | Retention time | Mass (m/z) | Family | Compound |
|---|---|---|---|---|
| **MRE** | 5.370 | 340.9 | Hydroxycinnamic acids | Caffeic acid 4-O-glucoside |
| | 32.517 | 476.8 | Methoxyflavonols | Isorhamnetin 3-O-glucoside |
| | 33.976 | 608.8 | Flavonols | Quercetin 3-O-xylosyl-glucuronide |
| | 35.209 | 358.8 | Hydroxycinnamic acids | Rosmarinic acid |
| | 38.064 | 652.7 | Flavonols | Quercetin 3-O-(6"-acetyl-galactoside) 7-O-rhamnoside |
| | 45.484 | 328.8 | Methoxyflavonols | 3,7-Dimethylquercetin |
| | 46.990 | 324.9 | Hydroxycinnamic acids | p-Coumaric acid 4-O-glucoside |
| | 49.049 | 344.8 | Phenolic terpenes | Rosmanol |
| **CRE** | 5.959 | 340.9 | Hydroxycinnamic acids | Caffeic acid 4-O-glucoside |
| | 26.868 | 386.9 | Lignans | Medioresinol |
| | 29.045 | 304.9 | Catechins | (+)-Gallocatechin |
| | 34.649 | 520.9 | Anthocyanins | Petunidin 3-O-(6"-acetyl-galactoside) |
| | 35.434 | 476.8 | Flavonols | Quercetin 3-O-glucuronide |
| | 38.740 | 358.8 | Hydroxycinnamic acids | Rosmarinic acid |
| | 49.815 | 324.9 | Hydroxycinnamic acids | p-Coumaric acid 4-O-glucoside |

Table 3. Compounds identified by HPLC of *Thymus vulgaris* extracts by conventional and microwave-assisted extraction.

| Extract | Retention time | Mass (m/z) | Family | Compound |
|---|---|---|---|---|
| **MTE** | 6.216 | 341.0 | Hydroxycinnamic acids | Caffeic acid 4-O-glucoside |
| | 31.406 | 387.0 | Lignans | Medioresinol |
| | 34.574 | 305.0 | Catechins | (+)-Gallocatechin |
| | 38.267 | 447.0 | Flavones | Luteolin 6-C-glucoside |
| | 39.609 | 595.0 | Flavonols | Quercetin 3-O-glucosyl-xyloside |
| | 41.540 | 302.9 | Dihydroflavonols | Dihydroquercetin |
| | 45.319 | 358.9 | Hydroxycinnamic acids | Rosmarinic acid |
| | 50.159 | 286.9 | Other polyphenols | Phlorin |
| **CTE** | 6.021 | 340.9 | Hydroxycinnamic acids | Caffeic acid 4-O-glucoside |
| | 20.011 | 358.8 | Lignans | Lariciresinol |
| | 23.108 | 336.8 | Hydroxycinnamic acids | 3-p-Coumaroylquinic acid |
| | 25.859 | 386.9 | Lignans | Medioresinol |
| | 27.782 | 304.9 | Catechins | (+)-Gallocatechin |
| | 33.675 | 446.8 | Flavones | Luteolin 6-C-glucoside |
| | 36.850 | 358.8 | Hydroxycinnamic acids | Rosmarinic acid |
| | 41.683 | 286.8 | Other polyphenols | Phlorin |
| | 45.805 | 270.8 | Other polyphenols | Arbutin |
| | 47.709 | 330.9 | Phenolic terpenes | Carnosic acid |

Bioassays of the extracts with *E. coli* and *S. aureus* were also performed. Figure 4 shows the qualitative results obtained for the antimicrobial load of *Rosmarinus officinalis* and *Thymus vulgaris* extracts against the selected microorganisms using the disk diffusion method. An antibiotic was used as a control, which showed the highest inhibitory effect compared to the tested extracts. Figure 5 presents the bar graph of the inhibition halos (mm) obtained for each of the extracts. According to the results of this test, the extracts showed good inhibition for *E. coli*, with inhibition halos of 12.68 (MRE), 13.29 (CRE), 11.15 (MTE), and 12.82 (CTE) mm. Whereas, for the *S. aureus* strain, halos of 13.52 (MRE), 13.87 (CRE), 10.45 (MTE), and 15.03 (CTE) mm were obtained.

Different researchers mention that gram-negative bacteria are less sensitive than gram-positive bacteria, therefore, these results can be attributed to the lipopolysaccharides in the cell wall of gram-negative bacteria, which may prevent the bioactive components present in the extract from reaching the membrane (Weerakkody et al., 2010)

Bonilla and coworkers, in 2016, performed bioassays against *E. coli* and *S. aureus*, of an ethanolic extract of rosemary obtained by the conventional method, resulting in a weak inhibition of this (Bonilla & Sobral, 2016). Furthermore, an ethanolic extract of thyme was tested by Mokhatari and collaborators against *E. coli* and *S. aureus* strains, finding an inhibition halo of 18.43 mm and 28.67 mm, respectively (Mokhtari et al., 2023).

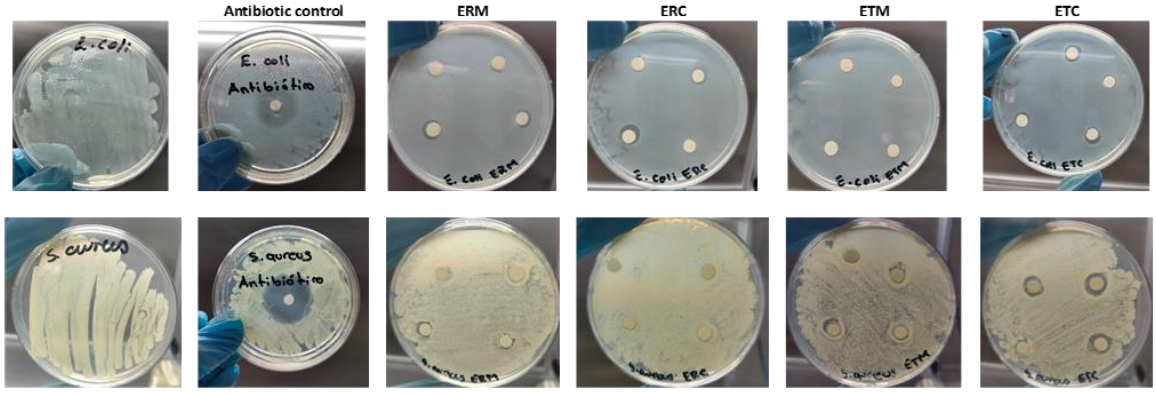


Figure 4. Inhibition of bacterial growth (*E. coli* and *S. aureus*) of *Rosmarinus officinalis* and *Thymus vulgaris* extracts.

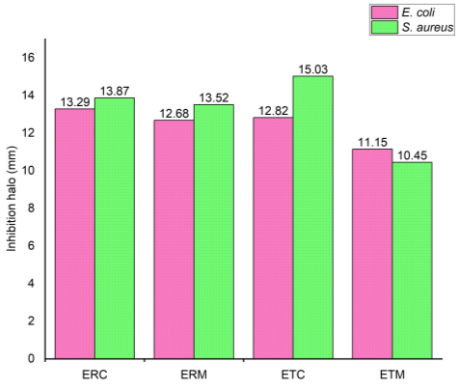


Figure 5. Inhibition halos (mm) of the extracts against *E. coli* and *S. aureus* strains.

## Conclusion

The extraction of bioactive compounds from two plants of the Lamiaceae family, rosemary (*Rosmarinus officinalis*) and thyme (*Thymus vulgaris*) was successfully carried out using a conventional and a microwave-assisted method. Employing FTIR-(ATR) and HPLC analysis, it was possible to identify the functional groups and compounds present in each of the extracts: rosmarinic acid, rosmanol, caffeic acid, luteolin, carnosic acid, and quercetin. In addition, the extracts showed decomposition temperatures above 200 °C. The MRE, CRE, and CTE extracts obtained better inhibition against *E. coli*, while for *S. aureus* it was the ETC extract. Therefore, according to the results obtained, it can be deduced that there is no difference between the extraction methods, except that CTE obtained a better inhibition against the strains analyzed.

## Acknowledgments

To CONAHCYT for the grant 1078907 awarded. As well as the support provided through the SEP-CONACYT Basic Sciences Project 2017-2018 CB2017-2018 A1-S-44977.

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