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**Formulation and biological evaluation of nanomedicins
with *Cenizo Leucophyllum frutescens* (BERL.) I.M.
JOHNSTON (Scrophulariaceae) extract against
*Mycobacterium tuberculosis***

Claudia Martinez-Rivas

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FORMULATION AND BIOLOGICAL EVALUATION OF NANOMEDICINS WITH CENIZO *Leucophyllum frutescens* (BERL.) I.M. JOHNSTON (SCROPHULARIACEAE) EXTRACT AGAINST *Mycobacterium tuberculosis*

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INDEX

I. ABSTRACT	5
II. RÉSUMÉ	5
III. INTRODUCTION	6
1. Tuberculosis	6
2. Natural Products	6
3. Polymeric nanoparticles	7
4. Encapsulation of natural compounds in polymeric nanoparticles	8
5. Drugs for the treatment of tuberculosis loaded in polymeric nanoparticles	9
IV. JUSTIFICATION	10
V. HYPOTHESIS	10
VI. GENERAL OBJECTIVE	10
VII. SPECIFIC OBJECTIVES	11
CHAPTER 1. Rifampicin and <i>Leucophyllum frutescens</i> : encapsulation in biodegradable polymeric nanoparticles and their biological evaluation against <i>Mycobacterium tuberculosis</i>	12
CHAPTER 2. Potential use of <i>Leucophyllum frutescens</i> as antioxidant agent and its encapsulation in polymeric nanoparticles	41
CHAPTER 3. Rifampicin and an active fraction of <i>Leucophyllum frutescens</i> loaded nanoemulsions, characterization and potential use against <i>Mycobacterium tuberculosis</i>	59
VIII. GENERAL CONCLUSIONS	73
IX. PERSPECTIVES	76
X. BIBLIOGRAPHY	78
APPENDIX I. PUBLICATIONS	93
APPENDIX 11. PARTICIPATION IN CONGRESSES	95

INDEX OF TABLES

<i>TABLE NO.</i>	<i>CONTENT</i>	<i>PAGE</i>
CHAPTER 1		
I	Percentage of yield of methanol extracts from leaves and roots of <i>Leucophyllum frutescens</i> and their fractions	23
II	Anti- <i>M. tuberculosis</i> activity H37Rv of the methanol extracts from leaves and roots of <i>Leucophyllum frutescens</i> and their fractions	25
III	Validation parameters of the chromatographic method for the quantification of rifampicin	28
IV	Validation parameters of the chromatographic method for the quantification of the vegetable samples from <i>Leucophyllum frutescens</i>	29
V	Characterization parameters of biodegradable polymeric nanoparticles containing rifampicin prepared by nanoprecipitation	30
VI	Characterization parameters of biodegradable polymeric nanoparticles containing methanol extract and hexane fractions from <i>Leucophyllum</i> prepared by nanoprecipitation	32
VII	Minimal inhibitory concentration (MIC) of biodegradable polymeric nanoparticles containing rifampicin (n=3)	34
VIII	Minimal inhibitory concentration (MIC) of biodegradable polymeric nanoparticles containing the vegetable actives (n=3)	35
IX	Minimal inhibitory concentration (MIC) of rifampicin with methanol extract and hexane fractions from <i>L. frutescens</i> roots against <i>M. tuberculosis</i>	36
X	Percentage of hemolysis of rifampicin, hexane fraction from roots of <i>Leucophyllum frutescens</i> and nanoparticles	38
CHAPTER 2		
I	Validation parameters of the four peaks-components of interest present in the ethanol fraction from <i>Leucophyllum frutescens</i> roots by HPLC	55
II	Polymeric nanoparticles characterization containing the ethanol fraction from <i>Leucophyllum frutescens</i> leaves	56
CHAPTER 3		
I	Characterization of nanoemulsions containing rifampicin and the hexane fraction from <i>Leucophyllum frutescens</i> roots	68

INDEX OF FIGURES

<i>FIGURE NO.</i>	<i>CONTENT</i>	<i>PAGE</i>
INTRODUCTION		
1	Structure of polymeric nanoparticles.	7
2	Preparation of polymeric nanoparticles by nanoprecipitation.	8
CHAPTER 1		
1	Chromatographic profile of methanol extract from leaves of <i>Leucophyllum frutescens</i> (EMH) and its fractions (FHH, HF1, HF2, HF3, HF4 y HF5) at 400 µg/mL analyzed by HPLC.	24
2	Chromatographic profile of methanol extract from roots of <i>Leucophyllum frutescens</i> (EMR) and its fractions (FHR, RF1, RF2, RF4 y RF5) at 400 µg/mL analyzed by HPLC.	24
3	Rifampicin chromatogram (30 ppm) obtained by HPLC (retention time=2 min).	27
4	MEB image of PLGA nanoparticles prepared by nanoprecipitation. A and D blank nanoparticles; B and E: nanoparticles with rifampicin; C and F: nanoparticles with the hexane fraction of <i>Leucophyllum frutescens</i> roots (A, B, and C: scale bar represents 1 µm; D, E and F: scale bar represents 500 nm).	33
5	Hemolytic activity of the vegetable samples from <i>Leucophyllum frutescens</i> : methanol extract and fractions ($\bar{x} \pm \sigma$, n=3).	37
6	Hemolytic activity of the encapsulated hexane fraction from roots of <i>Leucophyllum frutescens</i> (RF1 NP) and non-encapsulated (RF1) ($\bar{x} \pm \sigma$, n=3).	38
CHAPTER 2		
1	Percentage of the antioxidant activity of the methanol extract from leaves of <i>L. frutescens</i> and its fractions ($\bar{x} \pm \sigma$, n=3).	51
2	Percentage of the antioxidant activity of the methanol extract from roots of <i>L. frutescens</i> and its fractions ($\bar{x} \pm \sigma$, n=3).	52
3	Chromatographic profile of the ethanol fraction from <i>Leucophyllum frutescens</i> leaves (700 µg/mL) obtained by HPLC, showing four peaks-components of interest (retention times: 15.10, 18.20, 22.60 and 27.80 min, respectively).	53
4	Calibration curve of the four peaks-components of interest present in the ethanol fraction from <i>Leucophyllum frutescens</i> roots ($\bar{x} \pm \sigma$, n=3).	54

5	Antioxidant activity of the ethanol fraction from leaves of <i>Leucophyllum frutescens</i> (HF4) in its non-encapsulated and encapsulated form in biodegradable polymeric nanoparticles (NP). Ascorbic acid (AA) was taken as an antioxidant control ($\bar{x} \pm \sigma$, n=3).	57
CHAPTER 3		
1	Effect of the sonication time on the hydrodynamic diameter ($\bar{x} \pm \sigma$, n=3).	65
2	Effect of the concentration of Tween 80 solution on the hydrodynamic diameter ($\bar{x} \pm \sigma$, n=3).	66
3	Effect of the ratio on the amount of oleic acid (oil phase) and 1% w/v of Tween 80 solution (aqueous phase) on the hydrodynamic diameter ($\bar{x} \pm \sigma$, n=3).	67
4	Size distribution of nanoemulsions by intensity percentage: (A) rifampicin loaded nanoemulsion; (B) hexane fraction from roots of <i>Leucophyllum frutescens</i> .	69
5	Chromatogram of rifampicin in water (9 $\mu\text{g}/\text{mL}$) obtained by HPLC: (A) water peak; (B) rifampicin peak, retention time = 3.2 min.	70
6	Chromatogram of the hexane fraction from roots of <i>Leucophyllum frutescens</i> (230 $\mu\text{g}/\text{mL}$) obtained by HPLC: (A) peaks of methanol.	70
7	Effect of the pH of the dispersion medium of the nanoemulsions on the zeta potential: blank nanoemulsions (NE), containing rifampicin (RIF-NE) and containing the hexane fraction from roots of <i>Leucophyllum frutescens</i> (RF1-NE) ($\bar{x} \pm \sigma$, n=3).	71
8	Stability of blank nanoemulsions (NE), containing rifampicin (RIF-NE) and containing the hexane fraction from roots of <i>Leucophyllum frutescens</i> (RF1-NE) ($\bar{x} \pm \sigma$, n=3).	71

I. ABSTRACT

Tuberculosis is an emergency disease worldwide, the emergence of resistant strains to the treatment has produced the use of natural products as alternative. Studies have shown that *Leucophyllum frutescens* extracts present antimicrobial effect, but the disadvantage is that the extracts are recovered in a vehicle that contains organic solvents. The preparation of polymeric nanoparticles (NP) involves the elimination of the solvent in which the active is solubilized, which makes possible to use them as vehicles for the administration of the extracts. Therefore, the aim of this study was to design and develop formulations of NP with an extract of *L. frutescens*, and rifampicin (RIF), in order to evaluate the *in vitro* biological activity against *M. tuberculosis*. Firstly, the methanolic extract of leaves and roots of *L. frutescens* and its fractions were obtained. The anti-*M. tuberculosis* activity was determined, being the root extract (EMR) and its hexane fractions (FHR and RF1) the most actives with a MIC of 100, 40 and 40 $\mu\text{g/mL}$, respectively. RIF, EMH, FHR and RF1 were incorporated into NP by nanoprecipitation. NP of ≈ 180 nm with homogeneous size distribution were obtained. The NP were evaluated on *M. tuberculosis*, being the formulation of NP-PLGA-RIF (MIC=0.10 $\mu\text{g/mL}$) and NP-PLGA-RF1 (CMI=80 $\mu\text{g/mL}$) with better activity. Finally, the anti-*M. tuberculosis* activity of the combined form formulations was evaluated, the combination of RIF with NP-PLGA-RF1 produced better behavior, reducing the MIC of both without showing toxic effect. The studies carried out in this work showed the potential use of an NP formulation contains a vegetable fraction of *L. frutescens* in combination with RIF as an alternative against *M. tuberculosis*.

II. RÉSUMÉ

La tuberculose est une maladie d'urgence dans le monde, l'apparition de souches résistantes au traitement a produit l'utilisation de produits naturels comme alternative. Des études ont montré que les extraits de *Leucophyllum frutescens* présentaient un effet antimicrobien, mais l'inconvénient est que les extraits sont récupérés dans un véhicule qui contient des solvants organiques. La préparation de nanoparticules polymériques (NP) implique l'élimination du solvant dans lequel l'actif est solubilisé, ce qui permet les utiliser comme véhicules pour l'administration des extraits. Par conséquent, le but de cette étude a été design et développer des formulations de NP avec un extrait de *L. frutescens* et de la rifampicine (RIF), afin d'évaluer l'activité biologique *in vitro* contre *M. tuberculosis*. Premièrement, l'extrait méthanolique de feuilles et de racines de *L. frutescens* et ses fractions a été obtenu. L'activité contre *M. tuberculosis* a été déterminée, l'extrait de racines (EMR) et ses fractions hexanique (FHR et RF1) ont été les plus actives avec une CMI de 100, 40 et 40 $\mu\text{g/mL}$, respectivement. RIF, EMH, FHR et RF1 ont été incorporés dans NP par nanoprecipitation. Des NP de ≈ 180 nm avec une distribution de taille homogène ont été obtenus. Les NP ont été évaluées sur *M. tuberculosis*, les formulations de NP-PLGA-RIF (CMI=0,10 $\mu\text{g/mL}$) et NP-PLGA-RF1 (CMI=80 $\mu\text{g/mL}$) ont montré la meilleure activité. Finalement, l'activité des formulations combinées contre *M. tuberculosis* a été évaluée, la combinaison de RIF avec NP-PLGA-RF1 a produit le meilleur comportement, réduisant la CMI des deux sans montrer un effet toxique. Les études réalisées dans ce travail ont montré l'utilisation potentielle d'une formulation de NP contient une fraction végétale de *L. frutescens* en combinaison avec le RIF comme alternative contre *M. tuberculosis*.

III. INTRODUCTION

1. Tuberculosis

Tuberculosis (Tb) is an infectious disease caused by *Mycobacterium tuberculosis* (OMS, 2018), which is an acid-fast bacillus. It particularly affects the lungs, developing pulmonary Tb (Ruiz-Manzano *et al.*, 2008). Since 1993, it is considered by the World Health Organization (WHO) as an emergency disease worldwide. In 2016, 10.4 million people became ill with tuberculosis and 1.7 million died from this disease (OMS, 2018).

The current therapeutic scheme against Tb is based on the administration of four first-line antibiotics: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and streptomycin or ethambutol, for 6 months (WHO, 2005). Specifically, RIF is the only bactericidal drug that has activity on different populations that the bacteria present in the organism.

One limitation of the treatment is that, due to its long duration many patients do not follow it adequately, which causes the appearance of drug-resistant strains. Therefore, to improve the pharmacological therapy and avoid the resistance of the agent, it is urgent to find alternatives that reduce the time of treatment, or substances with a mechanism of action different to the existing drugs.

2. Natural Products

The plants have been used in traditional medicine to treat several diseases. WHO estimates that in many developed countries, from 70 to 80% of the population has tested a kind of alternative or complementary medicine (OMS, 2008).

Research in natural products has grown in recent years, numerous medicinal plant compounds that exhibit bioactivity have been described, including antimycobacterial activity (Camacho-Corona *et al.*, 2009).

For this reason and in response to the urgency of obtaining new drugs against strains of *M. tuberculosis*, several studies based on natural products have been developed. Some plants of the north of Mexico have been evaluated with favorable activity, among them, extracts of *Leucophyllum frutescens*, commonly named “cenizo”.

In the study by Molina-Salinas *et al.*, extracts of *L. frutescens* against drug-resistant and drug-resistant strains of *M. tuberculosis* were tested. The extract of the leaves and roots were obtained by maceration. The evaluation by microdilution in broth showed an activity of the root extract of 62.5 µg/mL, for the sensitive and the resistant strain. For the leaves, a MIC of 125 µg/mL was obtained. This study supports that, the selection of plants by ethnobotanical criteria increases the possibility of finding species with activity, not only against *M. tuberculosis*, but also against other microorganisms (Molina-Salinas *et al.*, 2007).

However, the disadvantage of the use of extracts is that they are recovered in vehicles containing organic solvents, therefore their application *in vivo* is not viable.

To overcome these limitations, new strategies have been proposed, including the use of polymeric nanoparticles (NP) as a vehicle for the administration of plant extracts.

3. Polymeric nanoparticles

The NP (Figure 1) are particles of less than 500 nm in diameter that are prepared from natural or synthetic, biodegradable or non-biodegradable polymers. Drugs, proteins, peptides or nucleotides can be incorporated into these polymeric systems for their therapeutic application.

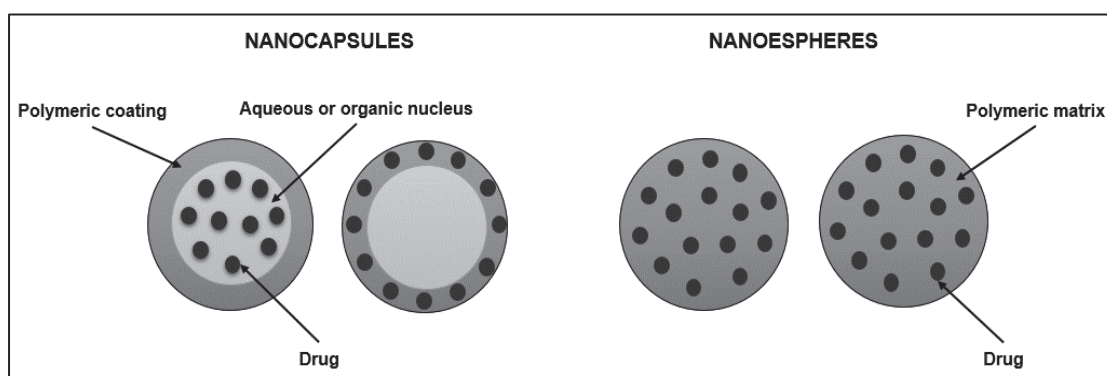


Figure 1. Structure of polymeric nanoparticles.

Among the features that stand out from these colloidal systems include the protection of the active, the targeting to an organ or cell and the sustained release of the encapsulated compound (Martínez Rivas *et al.*, 2017), this last virtue allows to reduce the frequency of

treatment dose. On the other hand, the preparation of NP involves the elimination of the solvent in which the active is solubilized (Figure 2), resulting in the encapsulation of the active compounds, free of organic solvents.

An additional advantage of NP is related to the versatility in their administration, depending their composition. NP can be applied topically (Martínez Rivas et al., 2017), oral or parenteral (Pilheu *et al.*, 2007).

Therefore, it is important to note that NP are a potential vehicle to increase the antimycobacterial activity of drugs used in the treatment of tuberculosis and to transport natural extracts that can not be administered directly to the body.

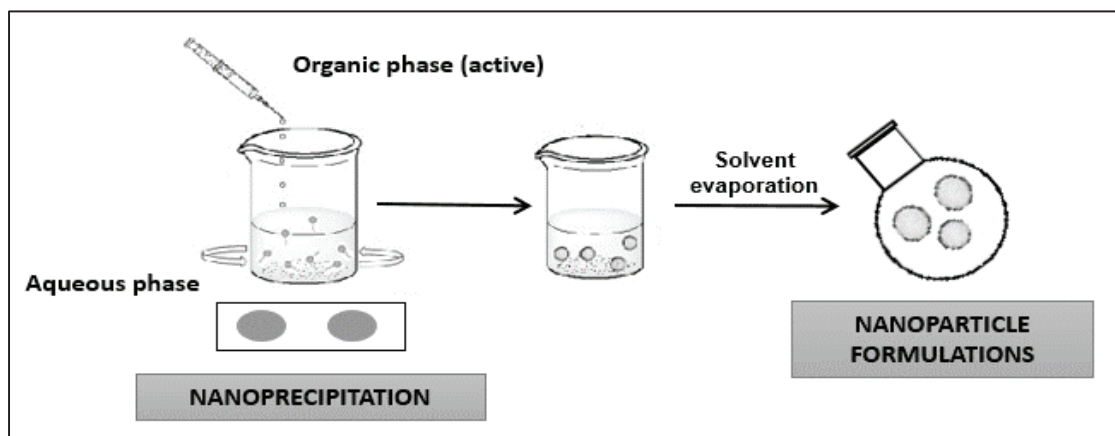


Figure 2. Preparation of polymeric nanoparticles by nanoprecipitation.

4. Encapsulation of natural compounds in polymeric nanoparticles

In recent years the interest in the encapsulation of plant extracts or derivatives has increased, an example is the encapsulation of resveratrol. Yao *et al.* incorporated the compound in chitosan NP with free amino groups on the surface. NP had a particle size of 487 nm and an polydispersity index of 0.144; in addition, chitosan NP showed sustained release *in vitro* (Yao et al., 2006).

In another study, Uthaman *et al.* extracted boswellic acid from the rubber resin of *Boswellia serrata*. The volumetric analysis showed a total of boswellic acid in the extract of 60%. Subsequently, formulations of NP were formulated with boswellic acid by nanoprecipitation method. NP had a diameter of 259 nm and a Z potential of -42.88 mV. The *in vitro* cytotoxicity assays were performed with different cancer cell lines (pancreas,

brain, prostate and melanoma). The PC3 prostate cancer cell line showed favorable cytotoxicity percentages. Therefore, they concluded that NP formulations containing boswellic acid can be used as potential anticancer agents in the treatment of prostate cancer (Uthaman *et al.*, 2012).

5. Drugs for the treatment of tuberculosis loaded in polymeric nanoparticles

Different studies have verified the protective properties (Calleja-Avellanal *et al.*, 2003), targeting (Yolandy *et al.*, 2010) and sustained release (Maksimenko *et al.*, 2010) from NP containing first-line drugs of the tuberculosis treatment, including RIF. NP improved the bioavailability of the drugs and reduced the frequency of the dose (Pandey *et al.*, 2003; Pandey y Khuller, 2006). In addition, there are several works that showed an effect against *M. tuberculosis* when they are encapsulated in NP. An example was observed in the study conducted by Yolandy *et al.*, who demonstrated targeting of isoniazid (INH) in nanoparticulate delivery systems. The *in vitro* tests were performed on THP-1 and U937 monocyte-macrophages and microscopic analysis showed that targeting was achieved. After 1 and 2 days of exposure of INH loaded NP, a significant inhibition in the growth of the microorganism was observed, but with a reduced value in the toxicity/efficiency ratio compared with free INH (Yolandy *et al.*, 2010). And, Booysen *et al.* proved that RIF and INH loaded NP have activity against the strain of *M. tuberculosis* H37Rv. In addition, they carried out the oral administration of the formulations in mice and observed a sustained release of the drugs for 7 days. They also determined the distribution of RIF and INH in the liver and the lungs for up to 10 days. With these results, they conclude that NP containing drugs for the treatment of tuberculosis have the potential to improve Tb chemotherapy (Booyesen *et al.*, 2013).

Therefore, the incorporation of plant compounds in NP as well as a first-line drug such as RIF is a promising alternative. In this context, the present work focused on the encapsulation of the extract and the fractions of *L. frutescens* in NP to generate a phytomedicine, as well as the incorporation of RIF, with the purpose of improving the activity against *M. tuberculosis*, and thus, proposing them as treatment for tuberculosis.

IV. JUSTIFICATION

The Tb is considered since 1993, by WHO, a worldwide emergency disease. One of the main causes of the great number of deaths, reported each year because of this disease, is due to the resistance that the bacillus strains have acquired in consequence to the inadequate monitoring of the treatment.

For this reason, it is urgent to find alternatives that reduce the treatment time and actives with a mechanism of action different from the existing drugs, to treat resistant strains. In recent years, natural products have been studied for the molecules with biological activity it contains, but their application is limited to the presence of organic solvents with which extracts are recovered.

The NP are a vehicle of biologically active compounds that offers multiple advantages, among them, the removal of the organic solvents in which the active compounds are dissolved. Therefore, the incorporation of plant compounds in NP is a promising alternative. In addition, NP provide sustained release to the encapsulated compound, making possible to reduce the dose frequency.

In this context, the present work is focused on incorporation of extracts and fractions of *Leucophyllum frutescens* plant to generate a phytomedicine, as well as encapsulation of RIF, in order to enhance the activity against *M. tuberculosis* and propose them as treatment for Tb.

V. HYPOTHESIS

The encapsulation of an extract or fraction of *L. frutescens* and rifampicin in polymeric nanoparticles allows the enhancement of the activity against *M. tuberculosis*.

VI. GENERAL OBJECTIVE

To design and develop NP formulations based on an extract or fraction of *L. frutescens* and RIF, in order to increase their biological activities against *M. tuberculosis*.

VII. SPECIFIC OBJECTIVES

- To obtain the methanol extracts of leaves and roots of *L. frutescens* by maceration and sonication and carry out their fractionation.
- To develop a chromatographic method validated by HPLC in order to characterize the extracts and their fractions and to obtain their chromatographic profiles.
- To encapsulate the extracts and active fractions by nanoprecipitation technique in NP and perform their physicochemical characterizations according to the size, polydispersity index (PDI), zeta potential, surface morphology, drug loading and encapsulation efficiency.
- To encapsulate RIF in NP by nanoprecipitation technique and perform their physicochemical characterizations according to the size, PDI, zeta potential, surface morphology and incorporated compounds.
- To evaluate the *in vitro* biological activity of the NP formulations on *M. tuberculosis*, as well as to determine the synergistic effect of the drug and the vegetable samples, free and encapsulated.
- To evaluate the toxicity of NP formulations with better antimycobacterial activities.

**CHAPTER 1. RIFAMPICIN AND *Leucophyllum frutescens*:
ENCAPSULATION IN BIODEGRADABLE POLYMERIC
NANOPARTICLES AND THEIR BIOLOGICAL EVALUATION
AGAINST *Mycobacterium tuberculosis***

ABSTRACT

Tuberculosis, is an emergency disease worldwide, caused by *Mycobacterium tuberculosis*. For its treatment four first-line drugs are administered; however, the emergence of resistant strains has led to the promising use of natural products as an alternative to combat the disease. Studies show that several plant extracts have action against *M. tuberculosis*, such as *Leucophyllum frutescens*. The disadvantage of the extracts is that they are recovered in vehicles as organic solvents, which makes difficult its application *in vivo*. The preparation of polymeric nanoparticles (NP) involves the elimination of the solvent in which the active is solubilized, it makes possible to use them as vehicles for the administration of extracts. While, RIF an antibiotic used in the treatment, is a model for the incorporation in NP. The objective of the present work was to formulate NP with the extract and fractions of *L. frutescens* and to evaluate its activity against *M. tuberculosis* in order to propose its use as a co-adjuvant of the antituberculosis drug RIF. In parallel, a formulation of RIF loaded NP was developed with the aim of improving the therapeutic performance of the drug. Firstly, chromatographic profile of methanol extracts of leaves and roots and their fractions of *L. frutescens* were obtained by HPLC. For leaves, four peaks-components were appreciated, for roots seven peaks-components were appreciated. The screening of methanol extracts of leaves and roots and their fractions for anti-*M. tuberculosis* activity revealed that the best activities were shown with the extract of roots (EMR) and the hexane fractions (FHR and RF1) with a MIC=100, 40 and 40 µg/mL, respectively. Subsequently, EMR, FHR, RF1 and RIF were encapsulated. NP had sizes between 140 and 190 nm, with homogeneous distributions and negative zeta potential. Then, the formulations were tested on *M. tuberculosis*. The formulation of RIF with the best activity was PLGA-PVAL-NP with a MIC of 0.10 µg/mL compared with the free drug that showed a MIC of 0.20 µg/mL. While, among the vegetable samples, were the formulations of FHR-PLGA-NP and RF1-PLGA-NP which

presented the best activities with a MIC of 80 µg/mL for both. An assay combining free actives and formulations were carried out. The combination of RIF and RF1-PLGA-NP reduced the MIC for both cases. Finally, the toxicity was measured by hemolysis, the results showed that EMR, FHR, RF1 free and formulated produced hemolysis but there is no presence of hemolysis with the combination of RIF and RF1-PLGA-NP. In this study, NP formulations containing RIF, active extract or active fractions of *L. frutescens* against *M. tuberculosis* were developed, finding promising the use of RF1-PLGA-NP as co-adjuvant of the antituberculosis drug RIF.

INTRODUCTION

Tuberculosis, since 1993, is considered by the World Health Organization as an emergency disease worldwide. In 2016, 10.4 million people became ill with tuberculosis and 1.7 million died (OMS, 2018). Four first-line antibiotics are administered to treat the disease, including rifampicin (RIF). However, the daily administration of the drugs for more than 6 months orally (NOM NOM-006-SSA2-1993).

One limitation of the treatment is its long duration, patients do not follow it adequately, which causes the appearance of resistant strains to the drugs. Therefore, to improve drug therapy and to avoid the resistance of the agent, it is urgent to find alternatives that reduce the time of treatment or find substances with a different mechanism of action from the existing drugs.

Plants have been used in traditional medicine to treat different diseases. WHO estimates that 70 to 80% of the population has ever resorted to one or another form of alternative or complementary medicine (OMS, 2008). The phytochemical study has grown in recent years and have been described numerous compounds obtained from medicinal plants that exhibit biological activity, including antimycobacterial activity described (Camacho-Corona *et al.*, 2009).

For this reason, and in response to the urgency of obtaining new treatments against *M. tuberculosis* strains, natural products studies have been developed. Several plants in northern of Mexico have a favorable activity against this bacterium. Particularly, the extracts of *L. frutescens* plant, commonly known as “cenizo”, were found among those that presents best activity.

Molina-Salinas *et al.*, evaluated the methanol extract from leaves and roots of *L. frutescens* on the sensitive and multi-drug resistant strain of *M. tuberculosis*. Leaves extract had no activity on the sensitive strain, whereas, in the resistant multi-drug strain showed a MIC of 125 µg/mL. Roots extract had a MIC of 62.5 µg/mL in both strains (Molina-Salinas *et al.*, 2007).

However, the disadvantage of this type of extracts is they are recovered in vehicles that contain organic solvents, thus their application *in vivo* is not viable. To overcome this limitation, new strategies have been proposed such as polymeric nanoparticles (NP) use. NP are systems that present sustained release allowing the reduction of the frequency of

treatment doses; in addition, NP confer to the active: protection (Yoo *et al.*, 2011), targeting to specific tissues and controlled delivery (Maksimenko *et al.*, 2010; Pandey y Khuller, 2006). For example, Yao *et al.*, carried out studies about the release of resveratrol (a compound present in grapes) incorporated in chitosan nanoparticles, the results showed that the nanoparticles had a sustained release and, therefore, a reduction in the frequency of the dose (Yao *et al.*, 2006).

Antitubercular drugs have also been incorporated in NP for the enhancement of their activities. Booyesen *et al.* encapsulated RIF and isoniazid (INH) in polylactic-*co*-glycolic acid (PLGA) NP covered with polyethylene glycol (PEG) by the double emulsion technique and spray drying. They obtained nanoparticle sizes from 230 to 380 nm with zeta potentials of +12.45mV and the encapsulation efficiencies for RIF and INH were 68.48 and 55.2%, respectively. In addition, they proved that RIF loaded NP had activity against the strain of *M. tuberculosis* H37Rv. On the other hand, they carried out the oral administration of a dose of the formulations in mice and observed a sustained release of the drugs for 7 days and biodistribution of RIF in the liver and INH in the lungs after 10 days. With these results, they concluded that PLGA NP containing antituberculosis drugs have the potential to improve Tb therapy (Booyesen *et al.*, 2013). For this reason, RIF being an antibiotic used in the treatment of Tb, is a model drug for its incorporation into NP.

The promising use of extracts or fractions is accompanied by the term biocompatibility. Two aspects that should be considered in the study of sample compatibility are: cytotoxicity and hemocompatibility. Knowing this last aspect, extracts or non-toxic fractions can be proposed for the use in an organism.

The aim of the present work was to take advantage of anti-*M. tuberculosis* activity of the extracts and fractions from *L. frutescens* plant to formulate them in NP in order to make viable its application against *M. tuberculosis*. In addition, the combined effect of extracts and fractions free and encapsulated was evaluated to propose the use of an extract or fraction of *L. frutescens* as co-adjuvant of the antituberculosis drug RIF.

MATERIALS AND METHODS

Preparation of *Leucophyllum frutescens* extracts and their fractions

Leaves and roots of *L. frutescens* were collected in Monterrey, N. L., Mexico in July 2013. Consequently, the plant material was washed, dried at room temperature and pulverized. An amount of 50 g of powdered leaves or roots were weighed and 350 mL of methanol was added. The extracts were obtained by ultrasound (Ultrasonic Cleaners, VWR Symphony, USA). For a period of 1 h, the temperature for the extraction was increased from 25 to 60 °C, once reached 60 °C, three cycles of 10 min of sonication and 10 min of rest were performed. Subsequently, the samples were filtered to obtain the solvent, which was evaporated under reduced pressure (Laborota 4003 control, Heidolph) in order to obtain the methanol extract of leaves (EMH) and roots (EMR).

With respect to the fractionation of the extracts, two methods were used. In the method 1, EMH was solubilized in methanol, it was placed in a separatory funnel and hexane was added; subsequently, both solvents were mixed and the hexane part was recovered and evaporated under reduced pressure in order to obtain the hexane fraction (FHH). The same procedure was followed with the EMR in order to obtain the hexane fraction from roots (FHR).

In the method 2, hexane was added to the EMH and stirred to aid the solubilization of the compounds related to the solvent, then, it was filtered to obtain the solvent and evaporated obtaining the hexane fraction called HF1. Chloroform was added to the solid insoluble in hexane, stirred and filtered to obtain the solvent with the compounds related to chloroform and evaporated for obtaining the chloroform fraction (HF2). This procedure was repeated with other solvents of higher polarity (ethyl acetate, ethanol and methanol) in order to obtain the HF3-HF5 fractions.

The fractionation method 2 was also performed to the EMR, identifying the obtained fractions as RF1-RF5. The yield (%) of the extracts and the fractions were calculated according to equations 1 and 2, respectively:

$$\text{Yield of the extract (\%)} = \frac{W_{\text{extract}}}{W_{\text{plant}}} \times 100 \quad (1)$$

$$\text{Yield of the fraction (\%)} = \frac{W_{\text{fraction}}}{W_{\text{extract}}} \times 100 \quad (2)$$

where, W_{extract} is the weight (g) of the obtained extract once the solvent is evaporated, W_{plant} is the weight (g) of the dried and powdered leaves or roots, W_{fraction} is the weight (g) of the obtained fraction once the solvent is evaporated, and W_{extract} is the weight (g) of the extract for the fractionation.

Chromatographic analysis of the extracts and fractions of *Leucophyllum frutescens*

For obtaining of the chromatographic profile of the EMH, EMR and their fractions, the samples were dissolved separately in acetonitrile (J. T. Baker, USA)/methanol (Tedia, USA), each solution was filtered through a 0.45 μm filter (Millipore, USA) to be analyzed by HPLC (VARIAN 9065, 9012, ProStar 410, USA). A Synergi™ 4 μm Fusion-RP 80 \AA (150 mm x 2.0 mm x 4 μm) column, a flow of 0.2 mL/min and 30 °C was used. The mobile phase was formic acid (purity: 90%, Millipore, USA) at 0.1% v/v and methanol with an isocratic elution of 45:55 for 40 min for leaves and 60 min for roots. The profiles were detected at $\lambda=215$ nm.

Chromatographic analysis of rifampicin

The RIF analysis (Sigma-Aldrich, USA) was carried out by HPLC using a Zorbax Eclipse XDB-C18 (150 mm x 2.1 mm x 5 μm) column, with a mobile phase formed by acetonitrile and water at 0.1% (v/v) of formic acid with an isocratic elution of 60:40 with a flow of 0.35 mL/min at 30 °C and at $\lambda=334$ nm.

Preparation of the calibration curve and method validation for the quantification of rifampicin

For the quantification of RIF a stock solution in acetonitrile was prepared. From this solution, the working solutions were prepared in a range from 5 to 30 $\mu\text{g/mL}$ and filtered through a 0.45 μm membrane. The analysis was carried out by HPLC to obtain the calibration curve.

The chromatographic method was validated through the variables: linearity, limit of detection (LOD) and limit of quantification (LOQ) according to the International

Conference on Harmonization (ICH). For the establishment of the linearity, the calibration curve was prepared and analyzed in triplicate. The LOD and LOQ were calculated by the following equations:

$$\text{Limit of detection (LOD)} = \frac{3.3 \sigma}{S} \quad (3)$$

$$\text{Limit of quantification (LOQ)} = \frac{10 \sigma}{S} \quad (4)$$

where, σ is the standard deviation of the response and S is the slope of the calibration curve.

The repeatability was established with three levels of concentration, six times each one the same day, while the reproducibility was obtained with three levels of concentration, six times each one in two different days.

Preparation of the calibration curve of the methanol extract from roots and their hexane fractions of *Leucophyllum frutescens*

For the quantification of the plant samples in NP, calibration curves of the EMR, FHR and RF1 were prepared. The samples were prepared in a mixture of acetonitrile/methanol at a concentration of 600 – 1700 $\mu\text{g/mL}$, the solution was filtered through a 0.45 μm filter to be analyzed by HPLC.

Synergi™ 4 μm Fusion-RP 80 Å (150 mm x 2.0 mm x 4 μm) column was used. The mobile phase was formic acid at 0.1% v/v and methanol in an isocratic elution of 45:55 for 60 min with a flow of 0.2 mL/min, 30 °C and at $\lambda=220$ nm.

The chromatographic method for the sample RF1 was validated through the variables: linearity, LOD and LOQ according to the ICH.

Encapsulation and characterization of rifampicin, methanol extract and hexane fractions of *Leucophyllum frutescens* roots in biodegradable polymeric nanoparticles

Four different formulations of RIF loaded NP were prepared by the nanoprecipitation technique proposed by Fessi *et al.* (1989). Briefly, the organic phase

was prepared containing polylactic acid (PLA; PURASORB) or polylactic-co-glycolic acid (PLGA; MEDISORB 85:15 DL) and 2 mg of RIF in 3 mL of a mixture of solvents (acetone:methanol). The organic phase was added to 10 mL of an aqueous phase, containing polyvinyl alcohol (PVAL) (Clariant, Mexico) or Lutrol F127 (BASF) as a tensoactive agent at a concentration of 1% (w/w).

Then, the organic solvent was evaporated under reduced pressure. In this way, four formulations of RIF loaded NP were obtained. The NP characterization was carried out determining the particle size and polydispersity index (PDI) by dynamic light scattering (Zetasizer Nano ZS90, Malvern Instruments, UK). The zeta potential was measured by electrophoretic light scattering (Zetasizer Nano ZS90, Malvern Instruments, UK).

Two different formulations EMR, FHR or RF1 in NP were prepared by the nanoprecipitation technique. The organic phase was prepared with the particle-forming polymer, 20 mg of PLA or 15 mg of PLGA, and the plant sample (8 mg for EMR or 4 mg for FHR or RF1) in solvent mixture (acetone: methanol). The organic phase was added to PVAL at 1% w/w in constant stirring. The NP characterization was carried out by determining the particle size, PDI and zeta potential.

To determine the drug loading and encapsulation efficiency (%L and %EE), the NP formulations were centrifuged at 25,000 rpm (Allegra 64R, Beckman Coulter, USA), the supernatant was decanted, and the pellets were lyophilized (Freeze Dry System, LABCONCO, USA). The lyophilized NP were dissolved in acetonitrile for NP-RIF and acetonitrile:methanol for NP-EMR, NP-FHR and NP-RF1.

The obtained solutions were analyzed according to the chromatographic method previously described to quantify the samples from the calibration curves of each one. Subsequently, the %L and % EE for each formulation were determined by the following equations:

$$\text{Drug loading (\%L)} = \frac{\text{Weight of the active in NP}}{\text{Weight of the NP pellet}} \times 100 \quad (5)$$

$$\text{Encapsulation efficiency (\%EE)} = \frac{\text{Weight of the active in NP}}{\text{Wight of RIF}} \times 100 \quad (6)$$

All the analyzes were carried out in triplicate, only the analysis of NP-PLA-EMR and NP-PLA-FHR was performed in duplicate. The experimental results were expressed as mean±standard deviation ($\bar{X}\pm\sigma$, n=3).

Analysis by Scanning Electron Microscopy

The surface morphology of the NP was observed through the scanning electron microscopy (SEM) (FEI Quanta 250 FEG) from the “*Centre Technologique des Microstructures*” (CT μ) of the Claude Bernard University Lyon 1, France. For the preparation of the samples, one drop of each NP suspension was deposited in a metal sample holder and dried at room temperature. Finally, the samples were plated with platinum under vacuum.

Anti-*M. tuberculosis* activity

The anti-*M. tuberculosis* activity was evaluated on the susceptible strain of *M. tuberculosis* H37Rv by the alamar blue assay in a microplate adapting the methodology used by Molina-Salinas *et al.* (Molina-Salinas *et al.* 2007). The strain was cultivated in Middlebrock 7H9 broth enriched with OADC (Becton Dickinson and Co., Sparks, MD, USA) at 37 °C for 14 days. After, the strain was adjusted according to the standard scale no. 1 of McFarland and diluted 1:20 for use in the assay. On the other hand, a stock solution in DMSO was prepared at a concentration of 20 mg/mL. Work solutions were prepared from the stock solution. On the other hand, in a 96-well microplate, 200 μ L of water was added to the wells in the periphery. While, in the work wells, 100 μ L of Middlebrock 7H9 broth enriched with OADC was added. Subsequently, in the first well of each row, 100 μ L of each previously prepared work solution was added. Once all the samples were added, serial dilutions 1:2 of each sample were performed, taking 100 μ L of the first well and adding them to the second well of the row, then, of the second well, 100 μ L was taken to add them to the third, this procedure was repeated until reaching the last well each row; the 100 μ L taken from the last well was eliminated. Finally, 100 μ L of the bacterial suspension was added to obtain a final volume in all wells of 200 μ L. The final concentrations for the EMH, FHR and RIF were from 0.7 to 400 μ g/mL; for RIF from 0.1 to 50 μ g/mL; for NP-RIF from 12.5 to 400 μ g/mL; for NP-EMR, NP-FHR and

NP-RF1 from 5 to 160 µg/mL. The microplates were incubated at 37 °C for 5 days. At day 5, 20 µL of the blue alamar reagent and 12 µL of 10% v/v of tween 80 were added to all the work wells, and the microplates were reincubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was determined from the color change of the blue to pink reagent. All tests were carried out at least in triplicate.

In a second stage of the study, the RIF was combined with the EMR, FHR and RF1 in order to determine the mixture with the best activity. The work carried out by Avijgan *et al.* (2014) was taken as a reference. Then, RIF was combined with the formulation of NP-RF1 and NP-RIF formulation with NP-RF1 to determine if there was a synergism in the antimycobacterial activity. To carry out the evaluation, the concentration of RIF was varied by column and the concentration of the vegetable samples was varied by row. For example, for the RIF and RF1 combination, the RIF concentration was varied in the 1st row and the volume of the solution was kept constant with RF1 for a final concentration of 40 µg/mL; in the 2nd row the concentration of RIF was varied, and the volume of the solution was kept constant with RF1 for a final concentration of 20 µg/mL and, thus, downwards.

All analyzes were carried out in triplicate, except for the combination of RIF with EMR (n=1).

Hemolytic activity assay

The assay of hemolytic activity was carried out according by UNE-EN ISO 10993-4. A blood sample, from a healthy patient, was placed in a tube with ethylenediaminetetraacetic acid (EDTA), as an anticoagulant. The sample was centrifuged, discarding the plasma and the white cell pack, and preserving only the red blood cells (RBC) package. The RBC were washed three times with PBS (pH=7.4) in a 1:1 ratio (RBC:PBS). After washing, the supernatant was discarded and the RBC were resuspended in PBS in a 1: 1 ratio (RBC:PBS) in order to obtain the working suspension of RBC. On the other hand, stock solutions of EMR and hexane fractions were dissolved in DMSO/PBS at a concentration of 1 mg/mL. Different concentrations of these samples were prepared from the stock solution, as well as NP formulations that showed the best activity against *M. tuberculosis* (NP-RIF and NP-RF1) in a range of 10 to 200 µg/mL in

DMSO from 0.1 to 2% v/v. The negative control to the hemolysis was prepared with 975 μL of PBS and the positive control to the hemolysis was prepared with 975 μL of milli-Q water. A 25 μL of the RBC suspension was added to all samples, including controls. The samples were incubated at 37 ° C for 1 h (VORTEMP 56, Labnet International, Inc., USA). After this time, the samples were centrifuged at 10,000 rpm for 10 min (Spectrafuge 24D, Labnet International, Inc., USA), recovering the supernatant of all the samples and adding them to a 96-well microplate. Finally, the absorbance of the supernatants was measure at $\lambda = 575$ nm in a microplate reader (Epoch, BioTek Instruments, Inc., USA). The hemolytic activity was expressed as a percentage according to equation 7:

$$\text{Hemolysis (\%)} = \frac{ABS_{\text{sample}} - ABS_{\text{neg}}}{ABS_{\text{pos}}} \times 100 \quad (7)$$

where, ABS_{sample} is the absorbance of the supernatant from the sample, ABS_{neg} is the absorbance of the negative control, and ABS_{pos} is the absorbance of the positive control. All analyzes were carried out at least in triplicate. The experimental results were expressed as $\bar{X} \pm \sigma$, n=3.

RESULTS AND DISCUSION

Yields of extracts of *Leucophyllum frutescens* and their fractions

The methanol extracts from the leaves and roots of *L. frutescens* were obtained by ultrasound. As shown in Table I, the highest yield was obtained with the leaves. In the study published by Sultana *et al.* the same tendency is observed, there was a higher yield in leaves than in roots. They obtained the methanol extracts from leaves and roots of *Moringa oleifera* by two extraction techniques, with an orbital agitator at room temperature and under reflux in a water bath for 6 h, obtaining a yield with *M. oleifera* leaves of 9.6 and 16.6%, respectively. The yields in roots were of 3.2 and 5.1%, respectively (Sultana *et al.*, 2009). Even, the yields for EMH is higher than EMR, 19.30 and 7.92%, respectively.

The ultrasound used during the extraction of EMH and EMR can facilitate the swelling and solvation causing the enlargement of the pores of the cell wall. The greater swelling will improve the rate of mass transfer and, occasionally, it will break the cell walls, which leads to an increase in extraction efficiency and/or reduction in extraction time (Huie, 2002). Soares-Melecchi *et al.* optimized the technique of extraction by ultrasound to obtain the extract from flowers of *Hibiscus tiliaceus* L. They weighed 5 g of flowers and added 150 mL of methanol (1:30 ratio), reaching the highest yield (17.10%) in 5 h of extraction (Soares Melecchi *et al.*, 2006). In our case, the ultrasound method developed to obtain the methanol extracts from leaves and roots of *L. frutescens* required less time (2 h). The percentages of yield of the fractions, with the methodology described above, are shown in Table I. When the hexane fraction is obtained, by any method, there was a higher yield in the hexane fractions from roots, it is possible to attribute greater number of related compounds in hexane by roots than leaves. While, increasing the polarity of the solvents, yields of leaves are observed higher than roots.

Table I. Percentage of yield of methanol extracts from leaves and roots of *Leucophyllum frutescens* and their fractions

<i>Leaves</i>	<i>EMH</i>	<i>FHH</i>	<i>HF1</i>	<i>HF2</i>	<i>HF3</i>	<i>HF4</i>	<i>HF5</i>
Yield (%)	19.30±4.20	3.46±0.09	2.55±0.31	6.10±4.34	1.17±0.28	33.05±3.09	3.48±0.12
<i>Roots</i>	<i>EMR</i>	<i>FHR</i>	<i>RF1</i>	<i>RF2</i>	<i>RF3</i>	<i>RF4</i>	<i>RF5</i>
Yield (%)	7.92±0.38	9.62±5.04	10.46±0.99	7.19±0.36	0.40±0.05	13.99±4.06	16.27±0.56

Chromatographic profiles of the extracts and fraction of *Leucophyllum frutescens*

The extracts and fractions from leaves and roots were analyzed by HPLC to obtain their chromatographic profile. The variability within the plants of the same species depends on the collection station and the origin of the plant, among other factors (Nguyen Hoai *et al.*, 2009). For this reason, the determination of the chromatographic profile is useful for the quality control of plant extracts (He *et al.*, 2015; Kim *et al.*, 2015; Xie *et al.*, 2007, Yang *et al.*, 2013).

The extract and fractions obtained from leaves of the plant reveal the presence of 4 main peaks (Figure 1), while the chromatographic profiles of the extract and fractions from roots reveal the presence of 7 main peaks (Figure 2).

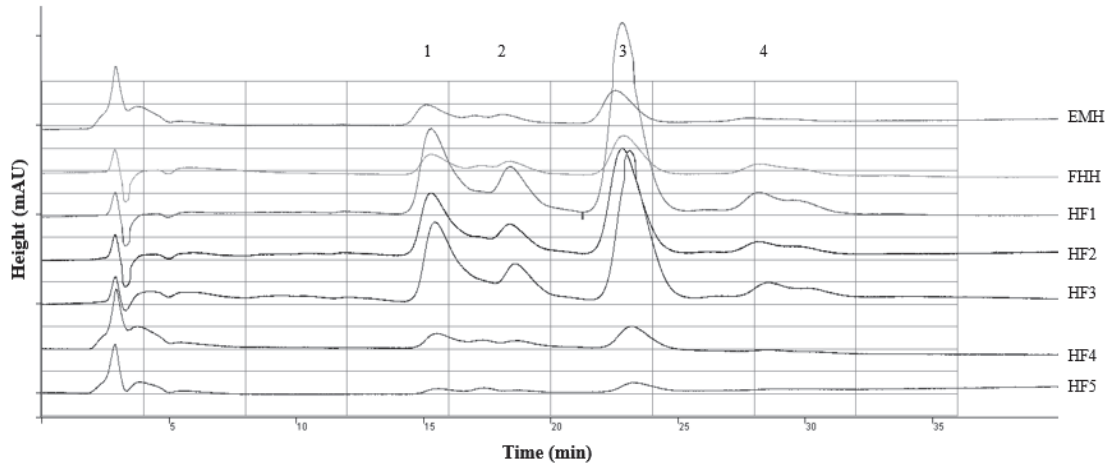


Figure 1. Chromatographic profile of methanol extract from leaves of *Leucophyllum frutescens* (EMH) and its fractions (FHH, HF1, HF2, HF3, HF4 y HF5) at 400 µg/mL analyzed by HPLC.

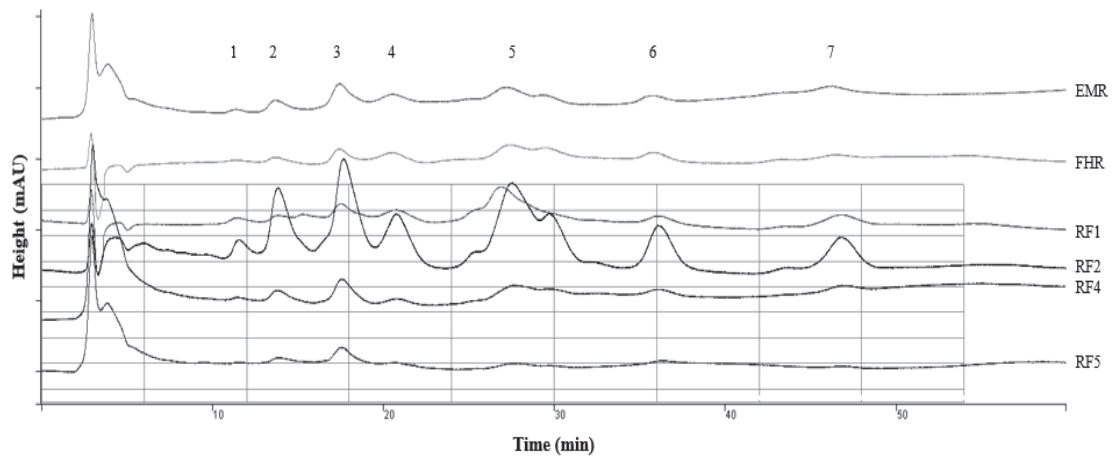


Figure 2. Chromatographic profile of methanol extract from roots of *Leucophyllum frutescens* (EMR) and its fractions (FHR, RF1, RF2, RF4 y RF5) at 400 µg/mL analyzed by HPLC.

Anti-*M. tuberculosis* activity of the extracts and fractions obtained from de *Leucophyllum frutescens*

The activity of extracts and fractions of *L. frutescens* against *M. tuberculosis* H37Rv was evaluated by the alamar blue microplate method. MIC obtained from the assay are presented in Table II. EMH had a MIC value above 200 µg/mL while EMR had a MIC value of 100 µg/mL. In the study by Molina-Salinas *et al.* similar trends are reported, because in leaves the tested concentrations did not show activity, while in roots MIC was of 62.5 µg/mL (Molina-Salinas *et al.*, 2007). Compared with our result, this variability in activity may be due to factors such as collection time or place of collection (Nguyen Hoai *et al.*, 2009).

Table II. Anti-*M. tuberculosis* activity H37Rv of the methanol extracts from leaves and roots of *Leucophyllum frutescens* and their fractions

<i>Leaves</i>	<i>EMH</i>	<i>FHH</i>	<i>HF1</i>	<i>HF2</i>	<i>HF3</i>	<i>HF4</i>	<i>HF5</i>
MIC (µg/mL)	>200	>200	>200	>200	>200	>200	>200
<i>Roots</i>	<i>EMR</i>	<i>FHR</i>	<i>RF1</i>	<i>RF2</i>	<i>RF3</i>	<i>RF4</i>	<i>RF5</i>
MIC (µg/mL)	100	40	40	200	200	>200	>200

The best activity against *M. tuberculosis* was collected with the hexane fractions from roots (FHR and RF1) at a MIC of 40 µg/mL. Comparing the methods of fractionation 1 and 2, FHR and RF1, respectively, both demonstrate the same efficiency to the extract compounds related to hexane with antituberculosis activity. Previously, the isolation and identification of a compound extracted from roots with potent anti-*M. tuberculosis* activity was reported (Molina-Salinas *et al.*, 2011), this fraction of interest was obtained with hexane, with a similar methodology to the fractionation method 1 performed in this work. However, an easy and rapid fractionation method was also developed (fractionation method 2), for the obtaining the fraction of interest preserving its antimycobacterial activity.

MIC achieved by FHR and RF1 is comparable to MIC reported in studies with other plants. Gemechu *et al.* investigated five medicinal plants in Ethiopia: roots of *Calpurnia aurea*, seeds of *Ocimum basilicum*, leaves of *Artemisia abyssinica*, *Croton macrostachyus*, and *Eucalyptus camaldulensis* used locally to fight tuberculosis. Methanol extracts of the plant materials were obtained by maceration and evaluated on *M. tuberculosis* strains such as: H37Rv, SIT73, SIT149, SB1176 and SB1953, which were comparatively the most susceptible to the extracts with MIC of ≤ 50 $\mu\text{g/mL}$ (Gemechu *et al.*, 2013). On the other hand, Nguta *et al.* evaluated the antimycobacterial activity of the hydroethanol extract from leaves of *Solanum torvum* *Aloe vera* var. *barbadensis*, *Dissotis rotundifolia*, *Chenopodium ambrosioides* and rizosimas of *Zingiber officinale* Roscoe on *M. tuberculosis* H37Ra and H37Rv. It was determined that the leaves of *S. torvum* were the best potential anti-*M. tuberculosis*, since, they showed a MIC of 156.3 and 1,250 $\mu\text{g/mL}$ against *M. tuberculosis* H37Ra and H37Rv, respectively (Nguta *et al.*, 2016). Likewise, Kahaliw *et al.* obtained the chloroform extracts from roots of different plants, among them *Pterolobium stellatum*, which showed the highest MIC. The fractionation of this extract was carried out by solvent partition, in order to obtain the hexane and ethyl acetate fractions. In this case, the activity given by the fractions was greater than the extract (Kahaliw *et al.*, 2017).

According to the results published with other plants, root fractions are potential plant samples against *M. tuberculosis*. Following the mentioned chromatographic profile (Figure 2), in roots there are more peaks than in leaves.

RF3 fraction was discarded for the determination of the chromatographic profile, antioxidant and hemolytic activity, due to experimentally a low percentage of yield and low anti-*M. tuberculosis* H37Rv were obtained.

Chromatographic analysis of rifampicin

Among the analytical methods, HPLC highlight, it allows the separation of the compounds according to their physiochemical characteristics, even if they are in a polymeric matrix (Moreno-Exebio y Grande-Ortiz, 2014).

Initially, the chromatographic conditions that allowed the detection of RIF were developed. Figure 3 showed that the molecule was detected at a retention time of 2.0 min.

This result is close to the obtained retention time by Glass *et al.*, who optimized a chromatographic method for the quantification of antituberculosis drugs. The retention time of RIF was 2.85 min using acetonitrile: tetrabutylammonium hydroxide (42.5:57.5, v / v) (0.2 mM) at a pH of 3.10 (Glass *et al.*, 2007).

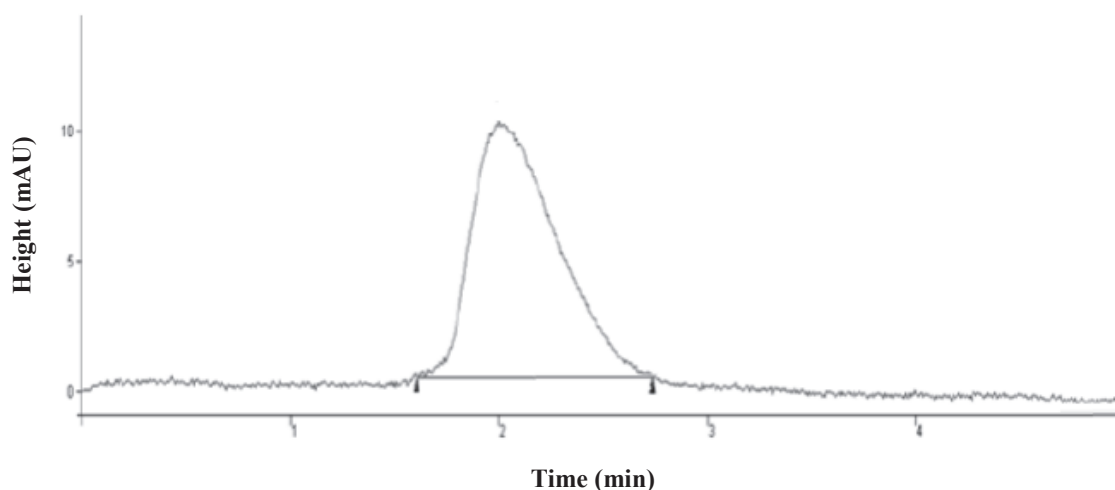


Figure 3. Rifampicin chromatogram (30 ppm) obtained by HPLC (retention time=2 min).

Preparation of the calibration curve and method validation for the quantification of rifampicin

The validation was carried out to ensure that the analytical procedure is accurate for the quantification of RIF. Table 3 shows the validation parameters established for the developed method. The concentration range (5-30 $\mu\text{g/mL}$) had linearity, with a correlation coefficient greater than 0.99. LOD was 0.71 $\mu\text{g/mL}$, while LOQ was 2.14 $\mu\text{g/mL}$. On the other hand, repeatability and reproducibility were measured, obtaining a variation of 5.97 and 6.79%, respectively. These values are acceptable, below the $\pm 15\%$ previously established (Glass *et al.*, 2007), therefore the method is accurate.

Table III. Validation parameters of the chromatographic method for the quantification of rifampicin

<i>Regression equation</i>	<i>Correlation coefficient (r)</i>	<i>LOD (µg/mL)</i>	<i>LOQ (µg/mL)</i>	<i>Repeatability (RSD, %)</i>	<i>Reproducibility (RSD, %)</i>
y = 8.8983x - 9.2698	0.99	0.71	2.14	5.97	6.79

Preparation of the calibration curve of the methanol extract from leaves and roots of *Leucophyllum frutescens*

As part of the characterization, from the chromatographic methods previously developed, the calibration curve of each plant sample (EMR, FHR and RF1) was analyzed in order to quantify the most abundant peaks in them, being selected the peak no. 5 in all the samples, and the peaks no. 6 and 7 in RF1. The method for RF1 quantification was validated by linearity, LOD and LOQ (Table IV).

For extracts or fractions from plants, the known compounds of the plant are selected, and the chromatographic method is developed for their quantification. For example, in the Amorim *et al.* study, three flavonoids were identified in the hydroethanol extract from the aerial part of *Tonina fluviatilis* (6,7-dimethoxyquercetin- 3-*O*-β-D-glucopyranoside, 6-hydroxy-7-methoxyquercetin-3-*O*-β-D-glucopyranoside and 6-methoxyquercetin-3-*O*-β-D-glucopyranoside) and a chromatographic method was developed and validated to quantify these compounds in the plant. For the preparation of the calibration curve, solutions of the three flavonoids were prepared and the regression equation for each was determined. LOD and LOQ of the calibration curves were below 30 µg/mL (Amorim *et al.*, 2014).

For RF1 with the developed method, LOD and LOQ are superior because internal marker compounds of the fraction are used, it was possible preparing solutions of different concentrations of this hexane fraction for the preparation of the calibration curve, instead of standard solutions. The validation was carried out to ensure that the analytical procedure is accurate for the quantification of RF1 once it was loaded in NP (Martínez-Rivas *et al.* 2017).

Table IV. Validation parameters of the chromatographic method for the quantification of the vegetable samples from *Leucophyllum frutescens*

<i>Active</i>	<i>No. Peak</i>	<i>Regression equation</i>	<i>Correlation coefficient (r)</i>	<i>LOD (µg/mL)</i>	<i>LOQ (µg/mL)</i>
EMR	5	$y = 1.712x - 355.49$	0.99	ND	ND
FHR	5	$y = 1.4231x - 591.75$	0.99	ND	ND
RF1	5	$y = 2.0497x - 105.57$	0.99	209.22	634.00
	6	$y = 0.3646x - 38.803$	0.99	427.35	1295.00
	7	$y = 1.3158 - 224.39$	0.99	103.98	315.10

(ND=not determined)

Encapsulation and characterization of rifampicin, methanol extract and hexane fractions from roots of *Leucophyllum frutescens* loaded in polymeric nanoparticles

Nanotechnology has emerged as a promising area to target the active to reservoirs such as macrophages (Nasiruddin *et al.*, 2017). *M. tuberculosis* resides for a long period of time in the alveolar macrophages of the lungs (Jain *et al.*, 2013).

Due to the NP size, they are a promising vehicle for distribution through the body and to reach the target cells or organs. Nahar and Jain prepared NP with sizes of 150-200 nm and negative zeta potential. Subsequently, they determined the targeting of Amphotericin B in PLGA NP conjugated with mannose and PEG to organs rich in macrophages (liver, kidney, spleen, lung and lymph nodes) in a bioavailability study. Swiss albino adult mice and males were injected with NP formulations, after the treatment they were sacrificed to analyze the organs. The results showed targeting of NP to organs rich in macrophages (Nahar y Jain, 2009). Comparing these results with our work, these characteristics of size and surface charge are favorable for their potential use. Table V describes the characterization parameters of RIF loaded NP. For the four formulations developed to encapsulate RIF, a particle size in a range of 140 – 180 nm was obtained, with homogeneous size distributions and negative zeta potential.

The surface charge measured by the zeta potential is important because it is related to the stability of NP and drug release profiles (Honary y Zahir, 2013). The use of some emulsifiers (surfactants) modifies the surface charge, although the emulsifiers used in this work are neutral (Mura *et al.* 2011), and their charge is modified depending on the pH of the medium. For example, PVAL has acetates groups that are negatively ionized as the medium change from pH 3 to pH 9 (Wiśniewska *et al.* 2016). Honary and Zahir prepared PLGA NP with two non-ionic surfactants, PVAL and Pluronic F68 (PF68). They obtained NP with negative zeta potential. The result obtained with PLGA-PVAL-NP was -5 ± 1 mV and with PLGA-PF68-NP was -24 ± 1 mV (Honary y Zahir, 2013), this formulation with PVAL presents a similar result compared with our work with this formulation (-5.57 ± 1.35 mV).

Table V. Characterization parameters of biodegradable polymeric nanoparticles containing rifampicin prepared by nanoprecipitation

<i>Polymer</i>	<i>Emulsifier agent</i>	<i>Size (nm)</i>	<i>PDI</i>	<i>Zeta Potential (mV)</i>	<i>%L</i>	<i>%EE</i>
PLA	Lut	142.8±15.4	0.210±0.042	ND	2.8±0.1	30.4±0.3
PLGA	Lut	163.0±4.6	0.113±0.019	-15.93±3.10	0.7±0.1	6.0±1.0
PLA	PVAL	178.5±4.1	0.168±0.045	-7.89±1.82	2.2±0.1	35.9±1.0
PLGA	PVAL	176.3±9.4	0.139±0.007	-5.57±1.35	0.5±0.1	4.0±0.5

(Lut: Lutrol F127 NF; PVAL: polyvinyl alcohol); ND=not determined

($\bar{x}\pm\sigma$, n=3)

Then, the formulations were analyzed by HPLC for the quantification of the drug, this HPLC method was developed to detect RIF without interference from the polymeric matrix. As seen in Table V, the encapsulation percentages changed according to the polymer and surfactant involved in particle formation. NP formulation with the highest encapsulation of RIF was PLA-PVAL-NP formulation, with an encapsulation efficiency of 35.9%, which indicates that 35.9% of total RIF is encapsulated in NP. While, the lowest encapsulation was PLGA-PVAL-NP. This difference is given, because the polymer PLA has lower hydrophilic affinity than PLGA (Dalpiaz *et al.*, 2016). Therefore, PLA is more

related to hydrophobic drugs such as RIF. NP preparation technique used in this work (nanoprecipitation) with the formulation parameters used, are favorable for formulating NP with RIF.

Due to the results shown in the studies against *M. tuberculosis*, EMR and its fractions FHR and RF1 were selected to be encapsulated in NP. Table VI describes the characterization parameters of NP with plant samples. For the two formulations that were developed to encapsulate the EMR, FHR or RF1, a particle size in a range of 160 – 190 nm was obtained, with homogeneous size distributions and negative zeta potential.

As already mentioned, NP from 150 to 200 nm with negative zeta potential have shown targeting towards the organs rich in macrophages such as the lungs (Nahar y Jain, 2009), NP with EMR, FHR or RF1 obtained in our work, are potential formulations to reach the lungs. Likewise, they have the characteristics for the internalization by the alveolar macrophages that contain the tuberculosis bacterium. Nicolete *et al.* observed that their blank PLGA NP with zeta potential of -17.2 ± 6.1 mV were internalized by J774 macrophages at 4 h. Therefore, they conclude that the size and surface chemistry of the particles influences their absorption (Nicolete *et al.*, 2011).

The peak no. 5 was selected to be quantified in NP, for being an abundant component in plant samples. The peak was encapsulated in all formulations, which indicates that the NP preparation method is favorable for the encapsulation of extracts or fractions extracted from *L. frutescens* plant. Some researchers have encapsulated plant compounds in NP such is the case of do Nascimento *et al.*, who encapsulated the red propolis extract in five formulations of poly- ϵ -caprolactone (PCL) NP and quantified five flavonoids (liquiritigenin, pinobanksin, isoliquiritigenin, formononetin and biochanin A) by UPLC. %EE of the flavonoids in NP had an average value of 75% (do Nascimento *et al.*, 2016). However, in this work the chromatographic method was designed to detect the components of interest of the extracts and fractions obtained from *L. frutescens* and using them as internal markers for their quantification in NP.

Table VI. Characterization parameters of biodegradable polymeric nanoparticles containing methanol extract and hexane fractions from *Leucophyllum* prepared by nanoprecipitation

<i>Active</i>	<i>Polymer</i>	<i>Size (nm)</i>	<i>PDI</i>	<i>Zeta Potential (mV)</i>	<i>No. Peak</i>	<i>%EE</i>
EMR	PLA	160.7±2.7	0.259±0.045	-9.79±036	5	78.10±11.67
	PLGA	176.0±7.0	0.150±0.018	-6.11±1.16	5	57.94±5.08
FHR	PLA	189.8±2.2	0.114±0.019	-11.23±1.86	5	21.65±1.60
	PLGA	171.5±10.6	0.127±0.025	-7.76±1.64	5	21.63±1.01
RF1	PLA	180.5±2.1	0.110±0.013	-10.77±2.69	5	83.82±3.61
					6	81.42±16.53
					7	83.42±8.16
	PLGA	170.3±4.4	0.094±0.012	-7.73±1.49	5	77.71±1.0
					6	55.47±10.86
					7	63.72±7.84

($\bar{X} \pm \sigma$, n=3 and n=2)

Finally, the surface morphology of the NP by MEB was determined. Figure 4 shows the micrographs obtained from the blank NP, RIF NP and RF1 NP. NP had a spherical shape and a smooth surface, with a size around 80 nm and homogeneous distribution in all formulations.

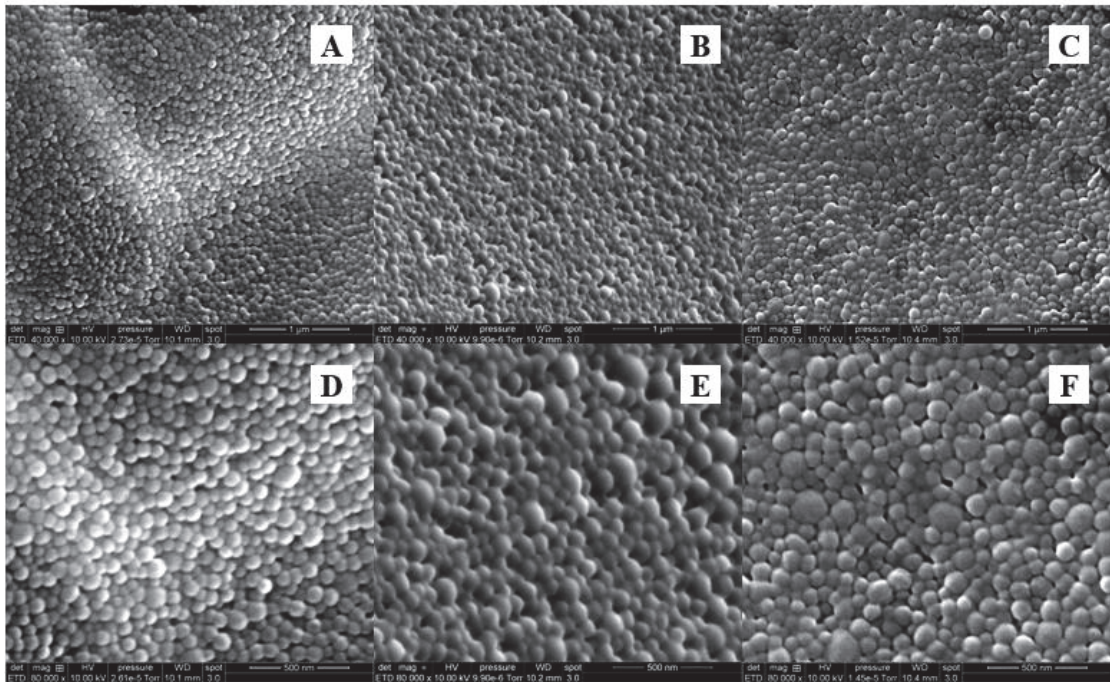


Figure 4. MEB image of PLGA nanoparticles prepared by nanoprecipitation. A and D blank nanoparticles; B and E: nanoparticles with rifampicin; C and F: nanoparticles with the hexane fraction of *Leucophyllum frutescens* roots (A, B, and C: scale bar represents 1 µm; D, E and F: scale bar represents 500 nm).

***Anti-Mycobacterium tuberculosis* activity of the nanoparticle formulations**

Once the formulations of NP were obtained, they were evaluated against *M. tuberculosis*. In Table VII, MIC of the formulations of NP with RIF are shown, the complete formulations (encapsulated and non-encapsulated active) and the formulations with the encapsulated active were evaluated. Likewise, MIC for RIF was determined (0.20 µg/mL). PLGA NP showed the best encapsulation, and it is the PLGA-PVAL formulation with encapsulated active that had the highest activity (0.10 µg/mL) even compared with the free drug. This enhancement in the antibacterial activity of the drug encapsulated in PLGA NP compared with its free form has already been observed in other studies.

As example is the work carried out by Darvishi *et al.*, who incorporated 18- β -glycyrrhetic acid (GLA) in PLGA NP with a size around 200 nm and negative zeta potential to test them against: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. MIC of NP with GLA and free GLA for *P. aeruginosa* was around 75 and 20 $\mu\text{g}/\text{mL}$, respectively; for *S. aureus* was of 75 and 35 $\mu\text{g}/\text{mL}$, respectively; and, for *S. epidermidis* was of 35 and 10 $\mu\text{g}/\text{mL}$, respectively. The results revealed that GLA NP had better activity than free GLA (Darvishi *et al.*, 2015).

Table VII. Minimal inhibitory concentration (MIC) of biodegradable polymeric nanoparticles containing rifampicin (n=3)

<i>Formulations</i>	<i>MIC total batch ($\mu\text{g}/\text{mL}$)</i>	<i>MIC encapsulated rifampicin ($\mu\text{g}/\text{mL}$)</i>
RIF-PLGA-Lut	0.20	0.30
RIF-PLA-PVAL	0.40	0.90
RIF-PLGA-PVAL	0.20	0.10

(Total batch: encapsulated and non-encapsulated rifampicin)

As mentioned, the best antimycobacterial activities were given by EMR, FHR and RF1 (MIC=100, 40 and 40 $\mu\text{g}/\text{mL}$, respectively), so they were selected to be formulated in NP. Table VIII shows the results of the assay for the determination of the activity of the NP formulations containing EMH, FHR or RF1, being FHR-PLGA-NP and RF1-PLGA-NP with the best activities. These results suggest that PLGA-PVAL-NP enhanced the activity against *M. tuberculosis* compared to free forms of RIF, FHR and RF1.

Table VIII. Minimal inhibitory concentration (MIC) of biodegradable polymeric nanoparticles containing the vegetable actives (n=3)

<i>Formulations</i>	<i>MIC total batch (µg/mL)</i>
EMR-PLA	400
EMR-PLGA	400
FHR-PLA	160
FHR-PLGA	80
RF1-PLA	160
RF1-PLGA	80

(Total batch: encapsulated and non-encapsulated active)

(MIC encapsulated active was not determined)

Subsequently, the combined effect between RIF and EMR, FHR or RF1 was evaluated, the best combination was achieved with RIF and RF1, MIC decreased from 0.20 to 0.10 µg/mL and 40 to 10 µg/mL, respectively (Table VIII). From this favorable combination, the combined effect of RIF with RF1-PLGA-NP and RIF-PLGA-NP with RF1-PLGA-NP was tested. The combination RIF-PLGA-NP with RF1-PLGA-NP did not have a better effect than the free combination. However, the combination of RIF with RF1-PLGA-NP, showed a better MIC for both, from 0.20 to 0.10 µg/mL and 80 to 20 µg/mL, respectively. It means, when RF1 is in NP, it is possible to enhance the effect of its free form. These results suggested that the combination of RIF with a hexane fraction from roots of *L. frutescens* against *M. tuberculosis* is favorable. The combination of drugs with natural agents increases the spectrum of activity and decreases the risk of emergence of resistant strains (Avijan *et al.*, 2014).

Table IX. Minimal inhibitory concentration (MIC) of rifampicin with methanol extract and hexane fractions from *L. frutescens* roots against *M. tuberculosis*

	<i>MIC of rifampicin</i> ($\mu\text{g/mL}$)		<i>MIC of vegetable samples</i> ($\mu\text{g/mL}$)
<i>EMR</i>	0.10	+	100
<i>FHR</i>	0.10	+	20
<i>RF1</i>	0.10	+	10
<i>RF1-PLGA-NP</i>	0.10	+	20

Hemolysis assay

One of the techniques for measuring compatibility can be through the determination of hemolysis (Fischer et al., 2003). The test of the extracts and their fractions was carried out according by UNE-EN ISO 10993-4 which establish that a percentage lower than 5% is considered non-hemolytic, considering the value of 100% to the total amount of hemoglobin present in the RBC sample added. RF5 showed no hemolysis at the concentrations analyzed. In contrast, RF2 and RF4 maintain a non-hemolytic effect at $\leq 100 \mu\text{g/mL}$, and EMR at $\leq 25 \mu\text{g/mL}$, Finally, the hexane fractions FHR and RF1 are non-hemolytic at $\leq 10 \mu\text{g/mL}$ (Figure 5).

Comparing the results of hemolytic activity with the *M. tuberculosis* activity, in EMR, FHR and RF1 there was presence of hemolysis at the established MIC (100, 40 and $40 \mu\text{g/mL}$, respectively). Vega Menchaca *et al.* evaluated the acute toxicity of the methanol extract from *L. frutescens* leaves. They carried out the evaluations in *Artemia salina*, reporting a LD_{50} of $196.37 \mu\text{g/mL}$, and in the VERO cell line, reporting an IC_{50} of $58.0 \mu\text{g/mL}$ (Vega Menchaca *et al.* 2013). These results suggest the presence of toxicity in the plant.

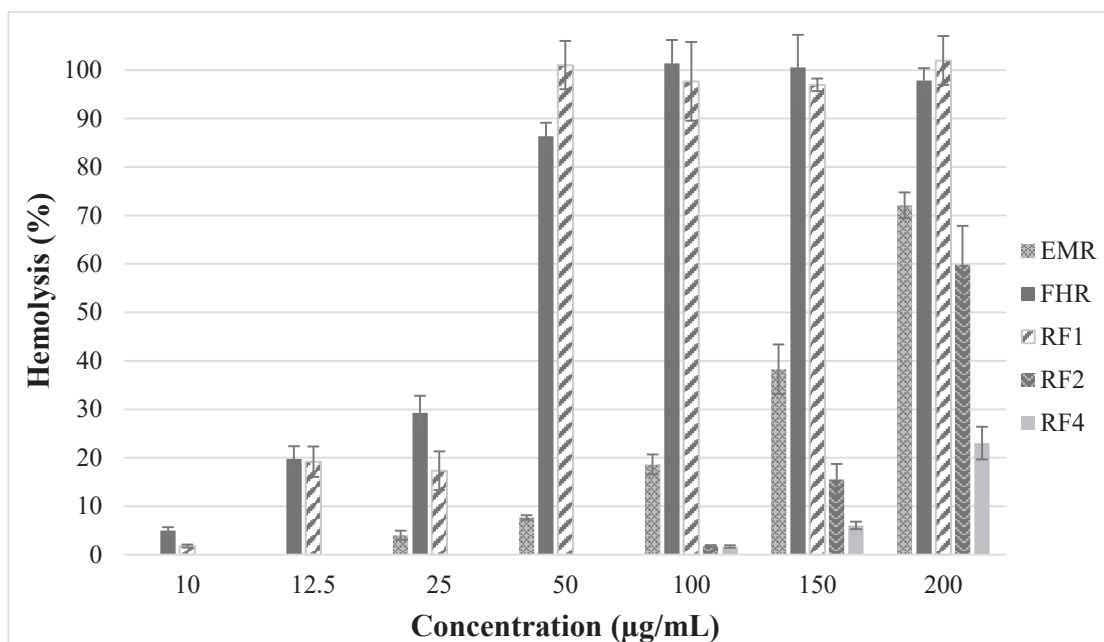


Figure 5. Hemolytic activity of the vegetable samples from *Leucophyllum frutescens*: methanol extract and fractions ($\bar{x} \pm \sigma$, n=3).

Also, the hemolysis percentage was determined as an indicator of the toxicity of RIF and NP formulations. For the concentration of RIF with activity against *M. tuberculosis* MIC were determined: *i*) free form (0.20 µg/mL), *ii*) in combination (0.10 µg/mL) and *iii*) RIF-PLGA-PVAL, being the formulation with the best activity. The results showed there was no presence of hemolysis. While, RF1 and RF1-PLGA-NP at the MIC against *M. tuberculosis* (40 and 80 µg/mL, respectively) are hemolytic. However, when RF1 is combined with RIF, the concentrations decreased from 40 to 10 µg/mL for RF1 and from 80 to 20 µg/mL for the NP formulation, and at these concentrations there was no presence of hemolysis (Figure 6). Additionally, Figure 6 showed that the encapsulation of RF1 in NP favors the reduction of its toxicity.

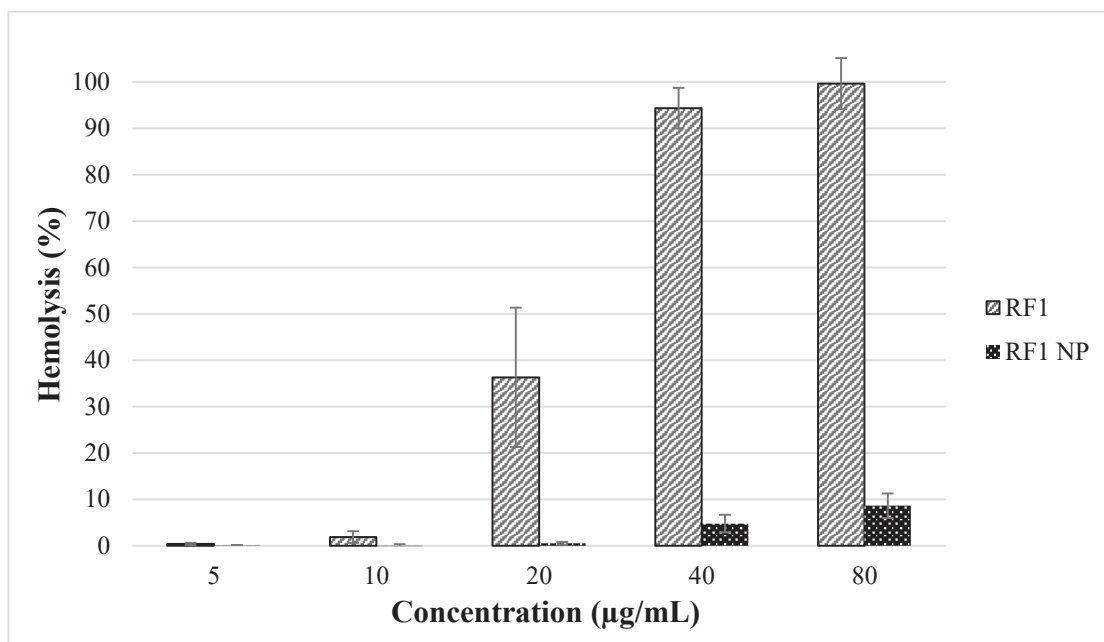


Figure 6. Hemolytic activity of the encapsulated hexane fraction from roots of *Leucophyllum frutescens* (RF1 NP) and non-encapsulated (RF1) ($\bar{x} \pm \sigma$, n=3).

Table X. Percentage of hemolysis of rifampicin, hexane fraction from roots of *Leucophyllum frutescens* and nanoparticles

<i>ANTITUBERCULOSIS ACTIVE</i>	<i>CONCENTRATION (µg/mL)</i>	<i>HEMOLYTIC ACTIVITY (%)</i>
RIF	0.20	N/H
RIF	0.10	N/H
RIF-PLGA-NP (E+NE)	0.20	N/H
RIF-PLGA-NP (E)	0.10	N/H
RF1	40	94.35±4.37
RF1	10	N/H
RF1-PLGA-NP (E+NE)	80	8.62±2.65
RF1-PLGA-NP (E+NE)	20	N/H

(E+NE=complete batch; E=encapsulated active)

($\bar{x} \pm \sigma$, n=3)

CONCLUSION

In this study several evaluations were carried out in order to have a broader knowledge about the biological activity of *L. frutescens* where is concluded:

- With the ultrasound method developed to obtain the methanol extract of leaves and roots of *L. frutescens* was obtained a yield of 19.3 and 7.92%, respectively, in a short time (2 h).
- Similar yields of the two fractionation methods of the hexane fractions were observed. However, method 2 was selected to continue with the following partitions because it is a faster and easier to execute.
- The chromatographic profiles by HPLC of the extracts and fractions revealed four and seven peaks of major components in leaves and roots, respectively.
- The bactericidal activity of the extracts and fractions against *M. tuberculosis* revealed that the roots are more active, specifically, EMR, FHR and RF1 with MIC of 100, 40 and 40 $\mu\text{g/mL}$, respectively. However, hemolysis occurred at these concentrations.
- Biodegradable NP of RIF and plant samples (EMR, FHR and RF1) of *L. frutescens* were prepared by nanoprecipitation, with a size from 140 to 190 nm, with homogeneous distributions and negative zeta potential.
- When RIF is encapsulated in PLGA-PVAL-NP enhanced its activity against *M. tuberculosis* (0.10 $\mu\text{g/mL}$) compared to the PLA-PVAL-NP and the free drug. The extract and fractions had a higher MIC when they are encapsulated. The most active formulations were with the fractions, showing better activity with PLGA NP (80 $\mu\text{g/mL}$) compared to PLA NP (160 $\mu\text{g/mL}$).
- The combination of plant samples with RIF decreased the MIC in both cases, demonstrating a better effect.
- At the active concentrations against *M. tuberculosis*, RIF and RIF loaded NP did not present hemolysis.
- The RF1 and RF1 loaded NP showed hemolysis at the active concentrations against *M. tuberculosis*. However, when RF1 is combined with RIF, the concentrations decreased from 40 to 10 $\mu\text{g/mL}$ for RF1 and from 80 to 20 $\mu\text{g/mL}$

for the NP formulation, and at these concentrations there is no presence of hemolysis.

Based on the results, the encapsulation of RF1 in NP decreased the toxicity. Therefore, a promising alternative for the treatment of Tb is the encapsulation of RF1 in NP and to combine it with conventional antituberculosis drugs.

CHAPTER 2. POTENTIAL USE OF *Leucophyllum frutescens* AS ANTIOXIDANT AGENT AND ITS ENCAPSULATION IN POLYMERIC NANOPARTICLES

ABSTRACT

Leucophyllum frutescens has been studied mainly for its activity against *Mycobacterium tuberculosis* but other biological properties in the plant have been less studied. In addition, for advance in the investigation of the plant to the application in some organism, it would be promising to transport the natural components in polymeric nanoparticles (NP), thus avoiding the use of organic solvents. It should be noted that NP have different advantages that improve the biological activity of the contained active. The objective of this work was to encapsulate an extract or fraction, with antioxidant activity, obtained from the methanol extract of *L. frutescens*. With nanoencapsulation, the vegetable sample is incorporated in a vehicle free of organic solvents, without losing its property, making possible to administer it in the body. First, the antioxidant activity of the extracts and fractions from leaves and roots of *L. frutescens* were evaluated to select the ethanolic fraction from leaves (HF4) as the most active. The chromatographic profile of the ethanol fraction of leaves was determined by High Performance Liquid Chromatography (HPLC), selecting four peaks-components for their use as internal markers in order to quantify them once the plant sample was encapsulated in NP. For quantification of the component peaks in NP, firstly, the chromatographic method was partially validated. The regression equations of the four peaks were obtained, with correlation coefficients greater than 0.99. In addition, the limit of detection and limit of quantification of each peak was determined resulting in 43.09 and 130.58, 38.62 and 117.03, 22.29 and 67.53 and, 45.86 and 138.97 $\mu\text{g/mL}$, for peaks 1, 2, 3 and 4, respectively. Suspensions of HF4 loaded NP were obtained by the nanoprecipitation method. NP had particle sizes around 200 nm with homogeneous distributions. While, the percentage of encapsulation of peaks 1, 2, 3 and 4 was 20, 23, 61 and 80%, respectively. Then, the antioxidant activity was evaluated using the oxidant peroxy, 2,2-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) in red blood cells. The fraction HF4 presented antioxidant activity at 100 $\mu\text{g/mL}$ and when HF4 was encapsulated

in NP, the activity was preserved. Finally, through the hemolysis test was concluded that HF4 and HF4-NP are not toxic to the active concentrations. Therefore, the potential use of HF4 loaded NP as an antioxidant agent is promising.

INTRODUCTION

Several compounds with biological activity from plants, have been isolated and identified. One of the most common examples is Taxol[®], which was obtained from the plant called *Taxus breuifolia* (Wani *et al.*, 1971). The large number of plants existing in the earth leads to the acquisition of new compounds or the promising discovery of activities not yet known in species not yet studied.

Leucophyllum frutescens, of the family Scrophulariaceae, is a plant commonly known as "cenizo". In Nuevo León it is the bush par excellence. In Texas it is called "shrub barometer" because its flowering depends on humidity in the environment and rainfall (Zaragoza, 2009). *L.* has been studied mainly for its activity against *Mycobacterium tuberculosis* (Alanís-Garza *et al.*, 2012; Molina-Salinas *et al.*, 2007, 2011). Some studies suggest other biological properties present in the metabolites of the plant.

However, a property not yet studied is its antioxidant capacity. Throughout the antioxidant effect, the body is protected from oxidative stress caused by free radicals. Free radicals play a fundamental role in different diseases such as cancer, inflammatory diseases or neurological disorders caused by age (Lobo *et al.*, 2010).

There are several studies focused on the investigation of the antioxidant activity of plants. Generally, the antioxidant properties are attributed to a chemical group called polyphenols (Özkan y Özcan, 2017). In the study by Saeed *et al.*, some extracts were obtained from the plant *Torilis leptophylla*, were studied, where the presence of phenols were found, which were related to their antioxidant activity. Likewise, an *in vivo* study suggested that methanol extract could be used against oxidative damage caused by carbon tetrachloride (CCl₄) by its antioxidant property (Saeed *et al.*, 2012).

To produce free radicals using the peroxy initiator, 2,2-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) in red blood cells (RBC) causes hemolysis, however, adding an antioxidant prevents hemolysis. Therefore, this system is an adequate model to determine the antioxidant activities of extracts or fractions of plants (An *et al.*, 2014). This method allowed to Jiang *et al.* to investigate the protection conferred by the aqueous extract of *Pueraria thomsonii* and *Pueraria lobata* on the RBC damage of rats caused by free radicals. No antioxidant activity was shown by the aqueous extract of *P.*

thomsonii, with an IC₅₀ around 1000 µg/mL. In contrast, the extract of *P. lobata* exhibited a potent activity, with an IC₅₀ of 194.0±6.9 µg/mL (Jiang *et al.*, 2005).

A limitation in the use of plant extracts with potential as antioxidant agents is on their *in vitro* or *in vivo* application because they are obtained in an organic solvent, which cannot be administered to an organism. A proposal to transport natural components is the use of polymeric nanoparticles (NP), because they show different advantages that improve the biological activity of the sample (Christofoli *et al.*, 2015).

For this reason, the aim of this work was to encapsulate an ethanol fraction obtained from the methanol extract of *L. frutescens* leaves, with antioxidant activity in order to contain it in a vehicle free of organic solvents without loss of activity, and thus to obtain a formulation with potential administration in an organism.

MATERIALS AND METHODS

Obtaining of the extracts of *Leucophyllum frutescens* and their fractions

Chapter 1 describes the procedure for obtaining the extracts from leaves and roots of *L. frutescens* and its fractions.

Determination of the antioxidant activity of the extracts and fractions from *Leucophyllum frutescens*

The antioxidant activity was measured by the damage of RBC producing free radicals with AAPH, the methodology described by Abajo *et al.* (2004). was used as reference. A blood sample, from a healthy patient, was placed in a tube with ethylenediaminetetraacetic acid (EDTA), as an anticoagulant. The sample was centrifuged, discarding the plasma and the white cell pack, preserving only the RBC package. RBC were washed three times with a solution of phosphates with salt (PBS, pH 7.4) in a ratio of 1:1 (RBC: PBS). After the washing, the supernatant was discarded, and the RBC were resuspended in PBS to subsequently dilute them 1:10 (RBC:PBS) in order to obtain the working suspension of RBC.

On the other hand, stock solutions of EMH, EMR and fractions in DMSO were prepared. Also, one solution of AAPH at 400 mM in PBS was prepared. Then, a volume

of each sample was taken to obtain the final concentrations in a range of 10 to 200 µg/mL in DMSO (0.15 to 3% v/v). The antioxidant control was ascorbic acid (AA), which was prepared at the same concentrations as the plant samples. 375 µL of the AAPH solution and 250 µL of the RBC suspension were added to all the samples.

The oxidant control was PBS, 375 µL of the AAPH solution and 250 µL of the RBC suspension. Two controls were added, one negative to the hemolysis (750 µL of PBS and 250 µL of RBC) and another positive to hemolysis (750 µL of milli-Q water and 250 µL of RBC). All test samples had a final volume of 1000 µL. Samples and controls were incubated at 37 ° C for 2.5 h with constant agitation (300 rpm) (VORTEMP 56, Labnet International, Inc., USA). After this time, the samples were centrifuged at 10,000 rpm for 10 min (Spectrafuge 24D, Labnet International, Inc., USA), recovering the supernatant of all the samples and adding them to a 96-well microplate. Finally, the absorbance of the supernatants at $\lambda=575\text{nm}$ was measured in a microplate reader (Epoch, BioTek Instruments, Inc., USA). The oxidative damage was determined by equation 1:

$$\text{Oxidative damage (\%)} = \frac{ABS_{\text{sample}} - ABS_{\text{ox}}}{ABS_{\text{pos}}} \times 100 \quad (1)$$

where, ABS_{sample} is the absorbance of the supernatant of the sample, ABS_{ox} is the absorbance of the supernatant of the oxidant control and ABS_{pos} is the absorbance of the supernatant of the positive control to the hemolysis. From this result the antioxidant activity was determined:

$$\text{Antioxidant activity (\%)} = 100 - \text{oxidative damage (\%)} \quad (2)$$

All analyzes were carried out at least in triplicate. The experimental results were expressed as mean±standard deviation ($\bar{x} \pm \sigma$, n=3).

Determination of the hemolytic activity of the extracts and fractions from *Leucophyllum frutescens*

The assay of hemolytic activity was carried out according by UNE-EN ISO 10993-4. A blood sample, from a healthy patient, was placed in a tube with

ethylenediaminetetraacetic acid (EDTA), as an anticoagulant. The sample was centrifuged, discarding the plasma and the white cell pack, and preserving only the red blood cells (RBC) package. The RBC were washed three times with PBS (pH 7.4) in a 1:1 ratio (RBC:PBS). After washing, the supernatant was discarded and the RBC were resuspended in PBS in a 1: 1 ratio (RBC:PBS) in order to obtain the working suspension of RBC.

On the other hand, stock solutions of EMR and hexane fractions were dissolved in DMSO/PBS at a concentration of 1 mg/mL. Different concentrations of these samples were prepared from the stock solution, as well as NP formulations that showed the best activity against *M. tuberculosis* (NP-RIF and NP-RF1) in a range of 10 to 200 µg/mL in DMSO from 0.1 to 2% v/v. The negative control to the hemolysis was prepared with 975 µL of PBS and the positive control to the hemolysis was prepared with 975 µL of milli-Q water. 25 µL of the RBC suspension was added to all samples, including controls. The samples were incubated at 37 ° C for 1 h (VORTEMP 56, Labnet International, Inc., USA). After this time, the samples were centrifuged at 10,000 rpm for 10 min (Spectrafuge 24D, Labnet International, Inc., USA), recovering the supernatant of all the samples and adding them to a 96-well microplate. Finally, the absorbance of the supernatants was measure at $\lambda = 575$ nm in a microplate reader (Epoch, BioTek Instruments, Inc., USA). The hemolytic activity was expressed as a percentage according to equation 3:

$$\text{Hemolysis (\%)} = \frac{ABS_{\text{sample}} - ABS_{\text{neg}}}{ABS_{\text{pos}}} \times 100 \quad (3)$$

where, ABS_{sample} is the absorbance of the supernatant from the sample, ABS_{neg} is the absorbance of the negative control, and ABS_{pos} is the absorbance of the positive control. All analyzes were carried out at least in triplicate. The experimental results were expressed as $\bar{X} \pm \sigma$, n=3.

Chromatographic analysis

For the obtaining of the chromatographic profile of HF4, it was dissolved in acetonitrile (J. T. Baker, USA)/methanol (Tedia, USA), the solution was filtered through

a 0.45 µm filter (Millipore, USA) to be analyzed by HPLC (VARIAN 9065, 9012, ProStar 410, USA). A Synergi™ 4 µm Fusion-RP 80 Å (150 mm x 2.0 mm x 4 µm) column, a flow of 0.2 mL/min and 30 °C was used. The mobile phase was formic acid (purity: 90%, Millipore, USA) at 0.1% v/v and methanol with an isocratic elution of 45:55 for 40 min for leaves and 60 min for roots. The profiles were detected at λ=224 nm.

Preparation of the calibration curve and method validation

HF4 was weighted and dissolved in methanol (Tedia, USA), to obtain a stock solution at 2000 µg/mL. From the stock, the working solutions were prepared in the range from 100 to 700 µg/mL and filtered through a 0.45 µm membrane (Millipore, USA), they were analyzed by the HPLC method before mentioned to obtain the calibration curve.

The chromatographic method was validated through the variables: linearity, limit of detection (LOD) and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH). For the establishment of the linearity, the calibration curve was prepared and analyzed in triplicate. The LOD and LOQ were calculated by the following equations:

$$\text{Limit of detection (LOD)} = \frac{3.3 \sigma}{S} \quad (4)$$

$$\text{Limit of quantification (LOQ)} = \frac{10 \sigma}{S} \quad (5)$$

where, σ is the standard deviation of the response and S is the slope of the calibration curve.

Encapsulation of the ethanol fraction from leaves of *Leucophyllum frutescens* in polymeric nanoparticles and its characterization

Once the validation parameters were established, NP containing HF4 were prepared by the nanoprecipitation technique proposed by Fessi *et al.* (Fessi *et al.*, 1989). Briefly, the organic phase was prepared dissolved 15 mg of polylactic-co-glycolic acid (PLGA; MEDISORB 85 15 DL) and 4 mg of HF4 in 3 mL of a mixture of solvents

(acetone:methanol). The organic phase was added to 10 mL of an aqueous phase, containing polyvinyl alcohol (PVAL) (Clariant, Mexico) as a stabilizer agent at a concentration of 1% w/w. Then, the organic solvent was evaporated under reduced pressure. The NP characterization was carried out determining the particle size and polydispersity index (PDI) by dynamic light scattering (Zetasizer Nano ZS90, Malvern Instruments, UK). To determine the drug loading and encapsulation efficiency (%L and %EE), the NP formulations were centrifuged at 25,000 rpm (Allegra 64R, Beckman Coulter, USA), the supernatant was decanted, and the pellets were lyophilized (Freeze Dry System, LABCONCO, USA). The lyophilized NP were dissolved in acetonitrile and methanol. The obtained solutions were analyzed by HPLC to quantify the peaks encapsulated from the calibration curve of each one, the results obtained were substituted in the following equations:

$$\text{Drug loading (\%L)} = \frac{\text{Weight of the active in NP}}{\text{Weight of the NP pellet}} \times 100 \quad (6)$$

$$\text{Encapsulation efficiency (\%EE)} = \frac{\text{Weight of the active in NP}}{\text{Weight of RIF}} \times 100 \quad (7)$$

All the analyzes were carried out in triplicate. The experimental results were expressed as $\bar{X} \pm \sigma$, n=3.

Determination of the antioxidant activity of the ethanol fraction from *Leucophyllum frutescens* leaves in polymeric nanoparticles

To evaluate the antioxidant activity of HF4 loaded NP, a suspension of RBC was prepared as previously described. For the preparation of the samples, solutions of AA and HF4 were prepared, the volume of the NP dispersion containing HF4 was taken to obtain the final concentrations of 25 to 200 $\mu\text{g/mL}$. 375 μL of the AAPH solution and 250 μL of the RBC suspension were added to all the samples. The antioxidant control was ascorbic acid (AA), which was prepared at the same concentrations of the plant samples. 375 μL of the AAPH solution and 250 μL of the RBC suspension were added to all the samples.

The oxidant control was PBS, 375 μL of the AAPH solution and 250 μL of the RBC suspension. Two controls were added, one negative to the hemolysis (750 μL of PBS and 250 μL of RBC) and another positive to hemolysis (750 μL of milli-Q water and 250 μL of RBC). All test samples had a final volume of 1000 μL . Samples and controls were incubated at 37 ° C for 2.5 h with constant agitation. After this time, the samples were centrifuged at 10,000 rpm for 10 min, recovering the supernatant of all the samples and adding them to a 96-well microplate. Finally, the absorbance of the supernatants at $\lambda=575\text{nm}$ was measured in a microplate reader. The results of the assay were determined by equation 1 and 2.

All analyzes were carried out at least in triplicate. The experimental results were expressed as $\bar{x}\pm\sigma$, n=3.

Determination of the hemolytic activity of the ethanol fraction from *Leucophyllum frutescens* leaves in polymeric nanoparticles

To evaluate the hemolytic activity of the HF4 in NP, a suspension of RBC was prepared as previously described. For the preparation of the samples, the necessary volume of the dispersion of NP containing HF4. The samples were tested in a concentration range from 25 to 200 $\mu\text{g}/\text{mL}$. The negative control to the hemolysis was prepared with 975 μL of PBS and the positive control to the hemolysis was prepared with 975 μL of milli-Q water. 25 μL of the RBC suspension was added to all samples, including controls. The samples were incubated at 37 ° C for 1 h. After this time, the samples were centrifuged at 10,000 rpm for 10 min, recovering the supernatant of all the samples and adding them to a 96-well microplate. Finally, the absorbance of the supernatants was measure at $\lambda=575$ nm in the microplate reader. The hemolytic activity was expressed as a percentage according to equation 3. The experimental results were expressed as $\bar{x}\pm\sigma$, n=3.

RESULTS AND DISCUSSION

Determination of the antioxidant activity of the extracts and fractions of *Leucophyllum frutescens*

Adding AAPH to RBC, free radicals are generated by oxidative damage in the lipids of the cell membrane, consequently, the hemolysis of the cell is induced. The hemolysis can be avoided with the presence of antioxidant substances. The extracts and fractions that exhibit antioxidant activity can protect the cell against oxidative damage, as a result, the RBC lysis is avoided. Paiva-Martins *et al.*, for example, made use of this method to determine the inhibition of the oxidative lysis of RBC. They obtained the compound called and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA) of olive oil and synthesized its metabolite 3,4-DHPEA-EDAH₂. Its objective was to induce the oxidative stress of RBC by AAPH, and to add the compounds obtained to measure the capacity of them to protect the cells. Both compounds showed RBC protection from oxidative hemolysis at concentrations ranging from 10 to 80 μM . 4-DHPEA-EDA achieved 70% of protection at 80 μM (Paiva-Martins *et al.*, 2015).

In the present study with *L. frutescens*, the samples with the best activity were obtained from leaves. The EMH, and the fractions HF1, HF2, HF3 and HF4 reached a protection around 80% from 50 $\mu\text{g/mL}$. While, the HF5 fraction reached this percentage from 100 $\mu\text{g/mL}$ (Figure 1).

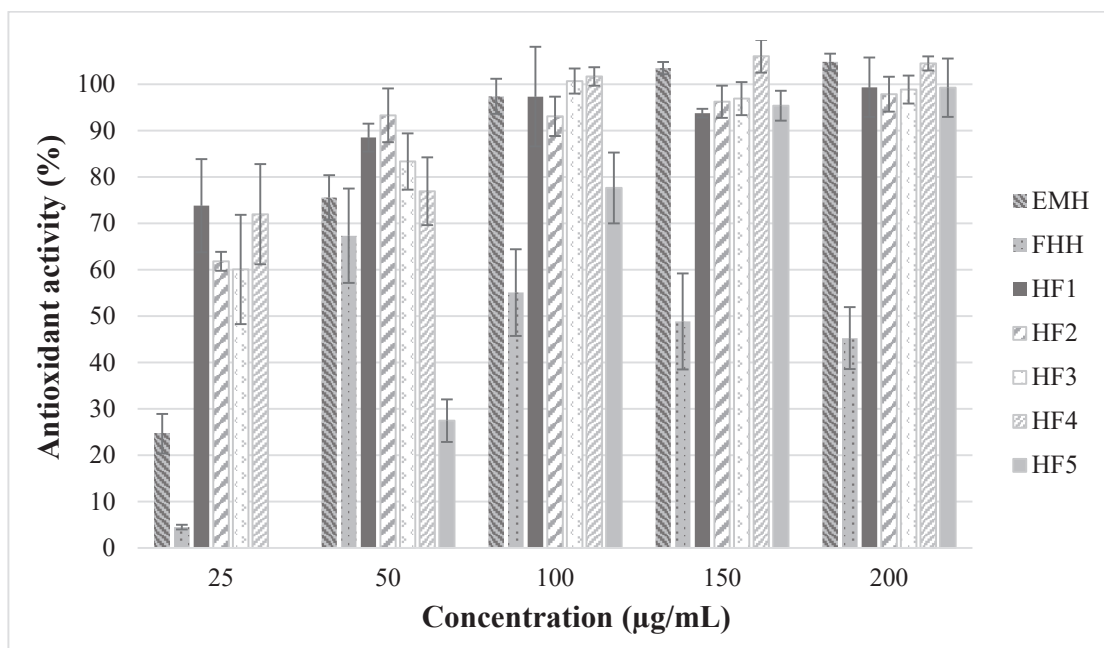


Figure 1. Percentage of the antioxidant activity of the methanol extract from leaves of *L. frutescens* and its fractions ($\bar{x} \pm \sigma$, n=3).

The ascorbic acid (AA) control presents antioxidant activity from 50 µg/mL (Figure 2). The results with *L. frutescens* revealed a protective effect at concentrations close to plants commonly reported with antioxidant activity. Karimi *et al.* investigated the aqueous and ethanol extracts of seeds of *Nigella sativa* L. and the aerial part of *Portulaca oleracea* L. in order to evaluate the cytoprotective effect of the extracts against the hemolytic damage induced by the free radical initiator AAPH. In general, the concentrations tested were from 25 to 1800 µg/mL, being from 150 µg/mL where an antioxidant effect is observed in the extracts (Karimi *et al.*, 2011). In contrast, FHH reached its highest protection capacity (around 70%) at 50 µg/mL, at higher concentrations this property decreases. This percentage decreases because at the same time, it has a hemolytic effect. Despite being obtained with the same solvent (hexane), the results for FHH and HF1 are different, therefore the fractionation method influenced the antioxidant activity of each fraction.

On the other hand, EMR reached 80% of protection from 25 to 50 µg/mL, however, when the tested concentration was 100 µg/mL, its antioxidant activity decreases (Figure 2).

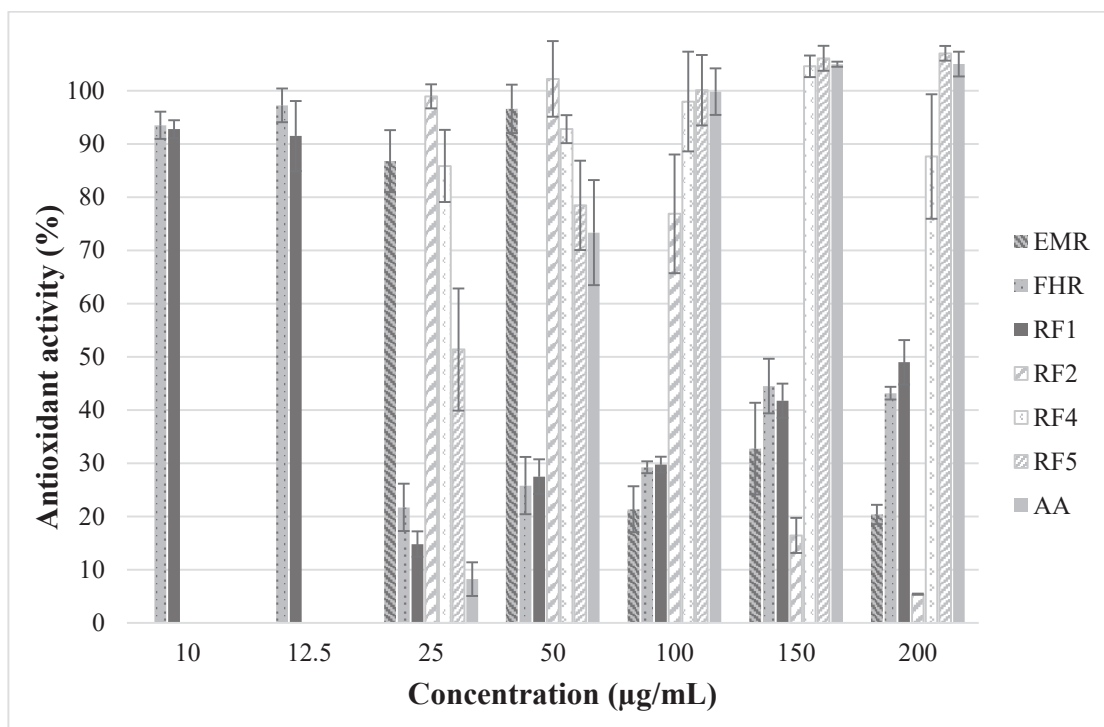


Figure 2. Percentage of the antioxidant activity of the methanol extract from roots of *L. frutescens* and its fractions ($\bar{x} \pm \sigma$, n=3).

As evidenced in Figure 2, the same behavior of EMR can be seen in its fractions. In the hexane fractions FHR and RF1, protection above the 80% is maintained in the range of 10 to 12.5 µg/mL, for RF2 and RF4 from 25-50 µg/mL and 50-150 µg/mL, respectively. In contrast, the antioxidant activity in RF5 is maintained from 50 to 200 µg/mL. Probably, the protection of the EMR, FHR, RF1, RF2 and RF4 samples decreased as presented in Chapter 1, they have a hemolytic effect, while in RF5 there is no presence of hemolysis. All these results demonstrated that, the EMH, HF1, HF2, HF3, HF4, HF5 and RF5, could be used in a range from 25 to 200 µg/mL and obtain an antioxidant effect, even, this effect can be comparable to AA control. Meanwhile, FHH, EMR, FHR, RF1, RF2 and RF4 could be used at specific concentrations.

Determination of the hemolytic activity of the methanol extract from leaves of *Leucophyllum frutescens* and its fractions

Because, extracts and fractions obtained from *L. frutescens* leaves showed greater antioxidant activity, we proceeded to the determination of toxicity by hemolysis.

The test of the extracts and their fractions was carried out according to UNE-EN ISO 10993-4, which states that a percentage lower than 5% is considered non-hemolytic. TEMH, HF1, HF2, HF3, HF4 and HF5 did not show hemolysis at the analyzed concentrations (25-200 $\mu\text{g} / \text{mL}$), while the FHH was not hemolytic below 25 $\mu\text{g}/\text{mL}$.

Chromatographic analysis

Of all the plant samples with antioxidant activity evaluated in the present work, HF4 fraction was selected to be encapsulated in NP. Firstly, it was analyzed by a HPLC to observe its chromatographic fingerprint or its peak profile, which is a useful tool for quality control, as well as to determine the amount of peaks or components present in the vegetable sample (Bian *et al.*, 2013).

The chromatographic method developed allowed the separation of four peaks or components of interest in HF4 (Figure 3). This method was validated in order to use it for the quantification of peaks 1-4, once the fraction in the NP was encapsulated.

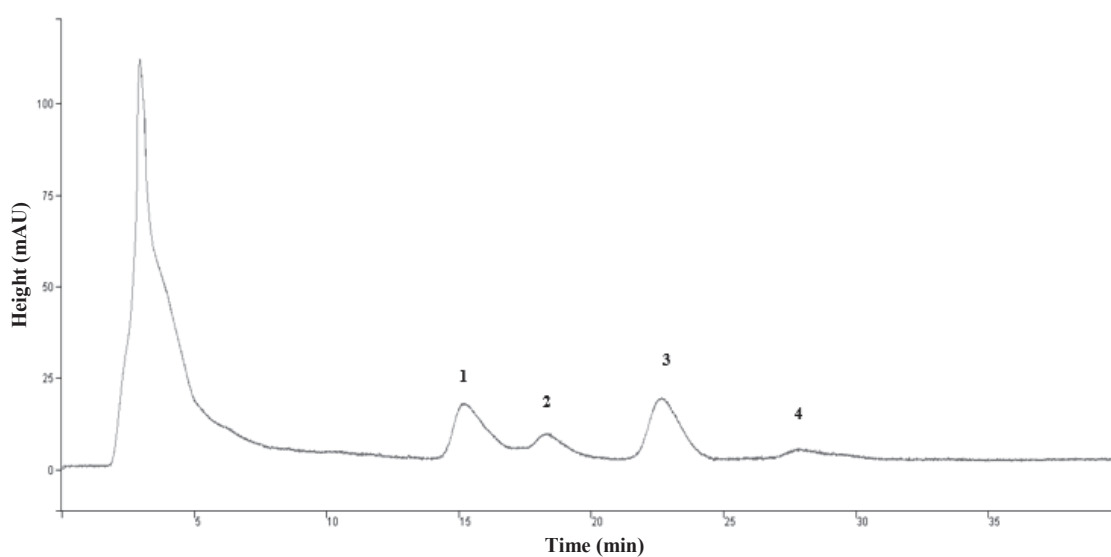


Figure 3. Chromatographic profile of the ethanol fraction from *Leucophyllum frutescens* leaves (700 $\mu\text{g}/\text{mL}$) obtained by HPLC, showing four peaks-components of interest (retention times: 15.10, 18.20, 22.60 and 27.80 min, respectively).

Calibration curve of HF4 and validation of the chromatographic method

The regression equation of each peak-component (1-4) was obtained from the calibration curve shown in Figure 4. As shown in Table 1, the correlation coefficients for the calibration curve of each peak-component were greater than 0.99. LOD and LOQ for each peak 1, 2, 3 and 4 were 43.09 and 130.58, 38.62 and 117.03, 22.29 and 67.53 and, 45.86 and 138.97 $\mu\text{g/mL}$, respectively.

In the area of natural products, when it is required to quantify a peak from a known molecule present in an extract, it is compared with the standard analyzed by the same chromatographic method. Such is the case of Assunção *et al.* who developed and validated a method by HPLC for the quantification of ellagic acid in the ethanol extracts from leaves of *Eugenia uniflora* L. (Myrtaceae) (Assunção *et al.*, 2017). In our work a chromatographic method was developed and validated by HPLC using peaks-components as internal markers for the quantification of HF4 in NP.

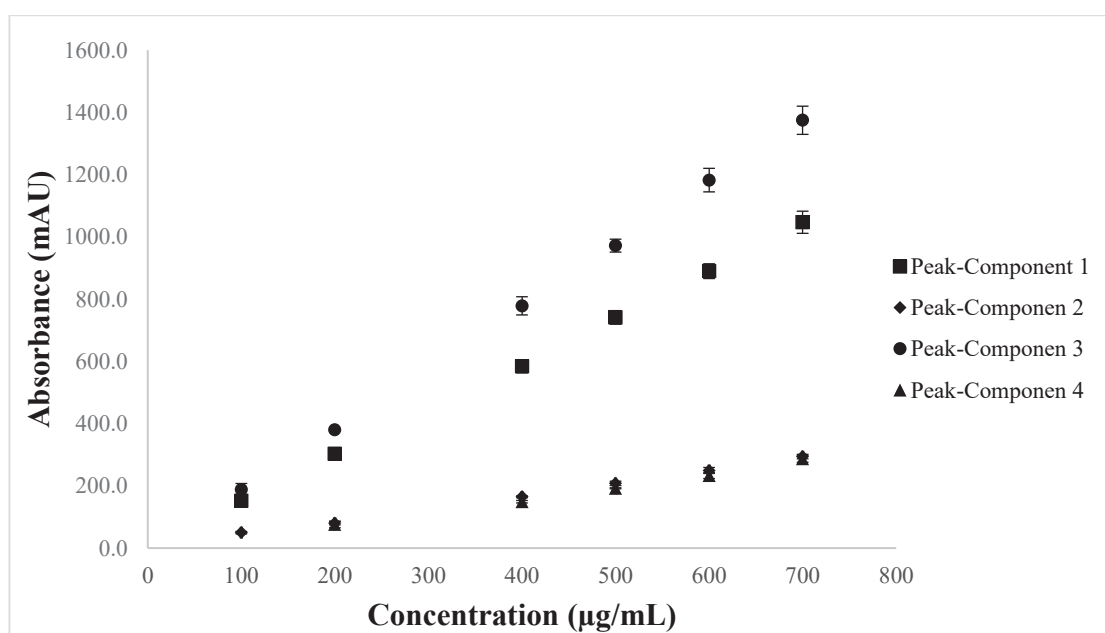


Figure 4. Calibration curve of the four peaks-components of interest present in the ethanol fraction from *Leucophyllum frutescens* roots ($\bar{x} \pm \sigma$, $n=3$).

Table I. Validation parameters of the four peaks-components of interest present in the ethanol fraction from *Leucophyllum frutescens* roots by HPLC

<i>Peak-Component</i>	<i>Regression equation</i>	<i>Correlation coefficient (r)</i>	<i>LOD (µg/mL)</i>	<i>LOQ (µg/mL)</i>
1	$y = 1.4826x + 1.8489$	0.99	43.83	130.58
2	$y = 0.4121x + 2.5638$	0.99	38.62	117.03
3	$y = 1.9841x - 14.17$	0.99	22.29	67.53
4	$y = 0.4178x - 13.207$	0.99	45.86	138.97

Encapsulation and characterization of the ethanol fraction from *Leucophyllum frutescens* leaves in polymeric nanoparticles

Once the method was validated, HF4 was encapsulated in NP by the nanoprecipitation method. The characterization of the formulation of NP with HF4 is shown in Table 2. NP sizes were obtained around 200 nm with a homogeneous size distribution (0.118). The lyophilized NP pellet with HF4 was dissolved in a mixture of acetonitrile: methanol, and the obtained solution was analyzed by HPLC. The area under the curve of each peak was replaced in its regression equation (Table 1), in order to obtain the concentration of the four component peaks in NP. Consequently, equations 6 and 7 were used to determine %L and %EE of peaks 1, 2, 3 and 4 in NP, being 4.13 and 19.83, 4.89 and 23.48, 12.81 and 61.52 and, 16.56 and 79.53%, respectively.

The encapsulation of the peaks increased as the hydrophobic nature of the peaks increased, the highest hydrophobicity was observed with peak 4 and therefore the highest encapsulation. Sanna *et al.*, obtained white tea extract by infusing the leaves in distilled water, after, it was encapsulated in poly-ε-caprolactone NP (PCL) and alginate as particle-forming polymers using the nanoprecipitation technique. The optimal formulation had a size around 380 nm and a unimodal distribution. They determined the %EE of two catechins, (-)-epigallocatechin gallate and (-)-epicatechin gallate, with values of 30.62 and 32.60%, respectively (Sanna *et al.*, 2015). In our work, higher %EE were determined, probably due to the nature of the components present in the *L. frutescens* fraction.

Table II. Polymeric nanoparticles characterization containing the ethanol fraction from *Leucophyllum frutescens* leaves

<i>Size (nm)</i>	<i>PDI</i>	<i>Peak-Component</i>	<i>Quantification</i>	
			<i>%L</i>	<i>%EE</i>
206.4±3.3	0.118±0.026	1	4.13±0.76	19.83±3.76
		2	4.89±0.86	23.48±4.25
		3	12.81±2.19	61.52±11.17
		4	16.56±3.21	79.53±16.21

($\bar{x} \pm \sigma$, n=3)

Determination of the antioxidant and hemolytic activity of the ethanol fraction from *Leucophyllum frutescens* leaves

The method used to determine the antioxidant activity of the HF4 NP was by the induction of oxidative damage of RBC with the reagent AAPH which generates hemolysis. Hemolysis of the RBC damage can be avoided with the presence of antioxidant substances. Figure 5 shows the antioxidant activity of HF4, HF4 NP and AA. HF4 fraction revealed around 80% of protection against oxidative damage (antioxidant activity) at 50 µg/mL. Achieving 100% antioxidant activity above 100 µg/mL. If this concentration is compared with the AA molecule evaluated as an antioxidant control, HF4 had better activity. When HF4 is encapsulated in NP, at 50 µg/mL the activity is around 80%; while 100% of antioxidant activity is reached at 100 µg/mL. In free HF4, total antioxidant activity is revealed at 100 µg/mL. Therefore, it is demonstrated that, although this fraction is formulated in NP, the antioxidant property is preserved.

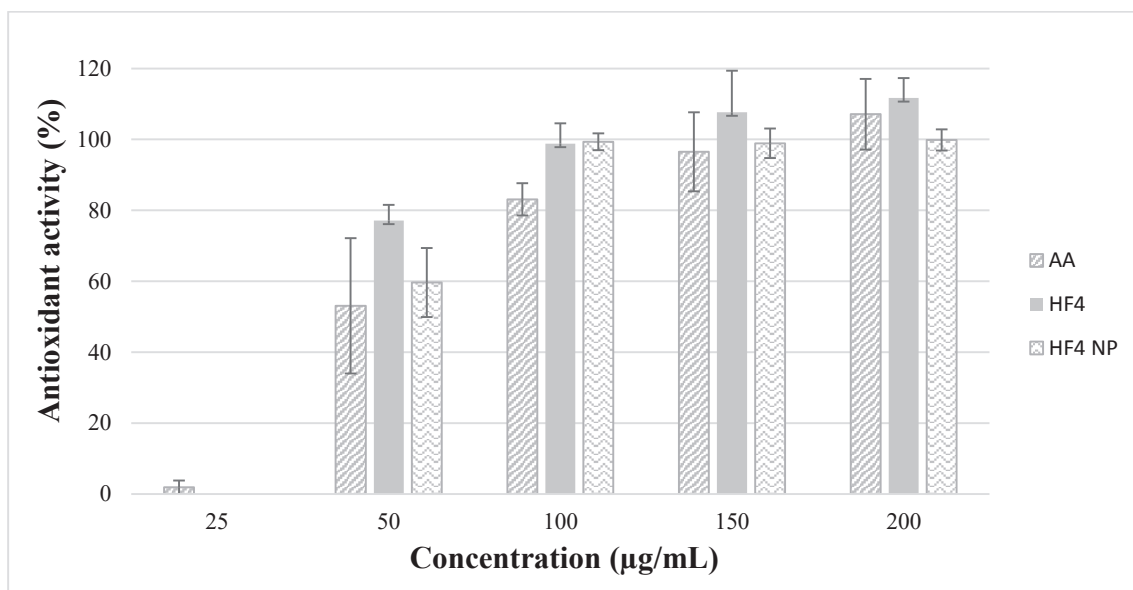


Figure 5. Antioxidant activity of the ethanol fraction from leaves of *Leucophyllum frutescens* (HF4) in its non-encapsulated and encapsulated form in biodegradable polymeric nanoparticles (NP). Ascorbic acid (AA) was taken as an antioxidant control ($\bar{x} \pm \sigma$, n=3).

In the aforementioned research by Sanna *et al.*, the antioxidant activity of the white tea extracts was evaluated by the *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The best activity of PCL NP was of DPPH, observed at 300 µg/mL with 60% of inhibition compared to the free extract that showed 100% of inhibition of DPPH at 25 µg/mL.

They attributed this difference to the low release of polyphenols from PCL NP (Sanna *et al.*, 2015). Less activity is obtained at a higher concentration if it is compared with our result, this may be due to the nature of the polymers, because PCL has a slower degradation rate compared with polylactics (Mahapatro y Singh, 2011), which is related to its low release. Likewise, it was evaluated by the hemolytic activity at active concentrations, determining that there is no presence of hemolysis. This test results in the potential use of HF4 fraction formulated or not formulated in NP for administration in the organism, however, it is in NP where the use of the organic solvents is avoided.

CONCLUSION

In the evaluation of the antioxidant activity of the extracts and fractions of *L. frutescens* is concluded:

- The extracts and fractions of *L. frutescens* plant showed protection, avoiding the toxicity lysis of RBC caused by the oxidation induced with the AAPH reagent. The FHH, EMR, FHR, RF1, RF2 and RF4 samples presented their maximum antioxidant activity at low concentrations.
- The antioxidant activity of EMH, HF1, HF2, HF3, HF4, HF5 and RF5 was showed from 25 to 200 µg/mL and non-hemolytic effect.
- In roots, the antioxidant activity of FHR and RF1 was more than 90% at 10 µg/mL and non-hemolytic effect. However, the activity was decreased when the concentration was increased due to hemolysis production. Similar effect was produced with other root samples.
- The HF4 fraction was selected for the studies that proceeded for its relevant antioxidant activity at a concentration of 100 µg/mL, similar to the reference antioxidant molecule AA.
- The chromatographic method was validated to be used for the quantification of major peaks-components in NP presented in the fraction and revealed a favorable incorporation of the *L. frutescens* fraction.
- The nanoprecipitation method for the preparation of NP loaded with HF4 was appropriate, a particle size around 200 nm with a homogeneous distribution was obtained, its properties are preserved, and it is non-toxic at the active concentrations.

CHAPTER 3. RIFAMPICIN AND AN ACTIVE FRACTION OF *Leucophyllum frutescens* LOADED NANOEMULSIONS, CHARACTERIZATION AND POTENTIAL USE AGAINST *Mycobacterium tuberculosis*

ABSTRACT

The interest on the use of nanoemulsions (NE) is related to its application as drug delivery systems. NE have shown several advantages such as the incorporation of hydrophobic drugs into a hydrophilic dispersion medium, targeting, cellular uptake and increased bioavailability. In recent years, special interest has been placed on the encapsulation of natural products such as extracts and essential oils obtained from plants to improve their biological effects. Among the plants is included *Leucophyllum frutescens* commonly named “cenizo”, which has been demonstrated an inhibition effect against *Mycobacterium tuberculosis*, causal agent of tuberculosis. For this reason, the aim of this work was to optimize and characterize a NE to encapsulate an active fraction from roots of *L. frutescens* (RF1), as well as, the antituberculosis drug rifampicin (RIF), and to evaluate their activity *in vitro* against *M. tuberculosis*. NE were prepared by ultrasonic emulsification and the influence of different preparation variables was tested. The results showed that at a longer time of sonication, the size and the polydispersity index (PDI) of NE globules decreased. The increase in the surfactant concentration, decreased the size and increased slightly the PDI. The increase in the amount of oil phase, increased the size and decreased slightly the PDI. The NE with a particle size around 180 nm and with homogeneous size distribution was used for the encapsulation of RF1 and RIF. The characterization of the loaded NE showed a particle size of 180 nm. For the determination of the percentage of the encapsulation efficiency of RF1, most abundant peaks were selected, being encapsulated at least 70%, while RIF was encapsulated at 99%. For the anti-*M. tuberculosis* activity were tested the blank NE, RF1-NE and RIF-NE, where, the blank NE showed inhibition at the same concentration as RF1-NE and RIF-NE, this behavior can be attributed to the presence of oleic acid as the oil phase. With this work the encapsulation and characterization of a vegetal sample (RF1) and RIF in NE are achieved. The optimal NEs characterized and tested are promising in the inhibition of *M. tuberculosis*.

INTRODUCTION

NE are systems thermodynamically unstable and kinetically stable are on nanometric scale (Gupta *et al.*, 2016). The interest for NE is related to the prospects of applications such disperse systems in medicine, in pharmaceutical and cosmetic industries (Koroleva y Yurtov, 2012). A nanoemulsion is constituted by oil, water and emulsifier (Gupta *et al.*, 2016). The emulsifier or emulsifying agent is a surfactant that reduces the interfacial tension between the immiscible phases, provide a barrier around the droplets and prevent coalescence of the droplets (Manoharan *et al.*, 2010).

The methods for the emulsion preparation are classified in high- and low-energy. The high-energy methods include mechanical shear such as that produced by high-shear stirring, ultrasonic emulsification, high-pressure homogenization. The low-energy method most widely used is phase inversion temperature (Koroleva y Yurtov, 2012).

They can be administrated by oral (Devalapally *et al.*, 2013), intranasal (Kumar *et al.*, 2008), topical (Hussain *et al.*, 2016) and parenteral routes (Araújo *et al.*, 2011). They can be formulated in variety of formulations such as foams, creams, liquids, sprays (Jaiswal *et al.*, 2015) or gels (Hussain *et al.*, 2016). As drug delivery systems studies have demonstrated many advantages such as incorporation of hydrophobic drug into a hydrophilic medium, targeting, cellular uptake and bioavailability.

A study carried out by Kumar *et al.*, has demonstrated it with an antipsychotic drug. A risperidone nanoemulsion (RNE) was prepared using capmul MCM as the oily phase and tween 80 as surfactant. A mixture of transcitol and propylene glycol was used as co-surfactant and distilled water as the aqueous phase. A risperidone mucoadhesive nanoemulsion (RMNE) was prepared by addition of chitosan. The globule size range was 15.5–16.7 nm. They proved the biodistribution of RNE, RMNE and risperidone solution (RS) in the brain and blood of Swiss albino rats by intranasal (i.n.) and intravenous (i.v.) administration. The brain/blood ratios of 0.617, 0.754, 0.948, and 0.054 of RS (i.n), RME (i.n), RMME (i.n) and RME (i.v), respectively, at 0.5 h are indicative of direct nose to brain transport bypassing the blood–brain barrier. Also, they obtained scintigraphy images following intravenous administration of RNE and intranasal administration of RNE and RMNE. The scintigrams demonstrate the accumulation of formulations in brain administered via respective routes. Major radioactivity accumulation was seen in brain

following intranasal administration of RMNE as compared to intravenous administration of RNE. For mucoadhesive nanoemulsions indicated more effective and best brain targeting of RSP. Added, significant quantity of risperidone was quickly and effectively delivered to the brain by intranasal administration of formulated. This study conducted in rats clearly demonstrated effectiveness of intranasal delivery of risperidone as an antipsychotic agent (Kumar *et al.*, 2008).

Another study by Hussain *et al.* reveal controlled and extended release profile and non-irritant properties of NE contained a broad-spectrum fungicidal antibiotic used primarily in the treatment of life-threatening systemic fungal infections. They prepared a NE and NE gel for topical delivery of amphotericin B (AmB) using sefsol-218 oil, Tween 80 and Transcutol-P. Then, NE was incorporate into the carbopol gel (1% w/w) formulation. The *in vitro* drug release for AmB NE was 42.12% and AmB NE gel was 10.96%. Formulation had shown 2.0- and 9.12-fold slower drug release, respectively, as compared to AmB solution (99.97%) in first 2 h suggesting controlled. The *in vitro* skin permeation study revealed NEs increase permeation rate. The cumulative amount of drug permeated at the end of 24 h was found to be 254.161 ± 1.45 mg, 870.42 ± 4.2 mg and 999.81 ± 7.3 mg for AmB DS, NE (pH 7.4) and AmB-NE gel, respectively. Also, the irritation potential of topical formulations was evaluated on Wistar albino rats. In this study, the results showed that no severe irritation symptoms such as erythema (redness) and edema (swelling) during 72 h except reference positive (Hussain *et al.*, 2016).

In recent years, special interest has been placed in the encapsulation of natural products as extracts and essential oils obtained from plants to enhance their biological effects (Blanco-Padilla *et al.*, 2014). Donsì *et al.* proved the activity of different essential oil components: carvacrol, limonene and cinnamaldehyde in the sunflower oil droplets of nanoemulsions. The antimicrobial activity was measured against three different microorganisms, such as *Saccharomyces cerevisiae* (ATCC 16664), *Escherichia coli* (ATCC 26) and *Lactobacillus delbrueckii sp. Lactis* (ATCC 4797). The microorganisms, centrifuged at 6500 rpm for 5 min at 4 °C, were resuspended in sterile distilled water to a final concentration of 10^4 CFU/mL in test tubes, where the nanoemulsions were added to the desired final antimicrobial concentrations. The test tubes were hence incubated at 32 °C for *S. cerevisiae* and *L. delbrueckii* and at 30 °C for *E. coli*. After 2 h and 24 h, the

surviving cells were evaluated by standard plate count method. The antimicrobial activity of formulations was evident over a longer time scale (24 h) (Donsi *et al.*, 2012). Tsai y Chen extracted catechins from tea leaf waste of *Camellia sinensis* (L.) Kuntze. The catechin extract was incorporated in NE and tested in human prostate cancer cell PC-3 and human fibroblast cell CCD-986SK. After 72 h incubation catechin nanoemulsion exhibited a lower toxicity toward CCD-986SK cells than catechin extract. While, catechin nanoemulsion had a major inhibitory effect in prostate cancer cell PC-3 proliferation with the IC₅₀ being 8.5 µg/mL than catechin extract with IC₅₀ being 15.4 µg/mL (Tsai y Chen, 2016).

Some studies have focused on the use of plants against the microorganism *M. tuberculosis*, causal agent of tuberculosis, with the aim of reducing the large number of new cases and mortalities that occur per year. The plant known as "cenizo" *L. frutescens* has been studied for this purpose. Molina-Salinas *et al.* determined that the methanol extracts from leaves and roots showed activity against *M. tuberculosis* (Molina-Salinas *et al.*, 2007). However, few compounds obtained from *L. frutescens* have been identified (Alanís-Garza *et al.*, 2012; Molina-Salinas *et al.*, 2011).

In this context, the aim of the study was to optimize and characterize of a NE to encapsulate an active fraction obtained from the methanol extract of roots of *L. frutescens*, as well as, the antituberculosis drug RIF, and the *in vitro* evaluation against *M. tuberculosis*.

MATERIALS AND METHODS

Preparation of *Leucophyllum frutescens* hexane fraction

Chapter 1 describes the procedure for obtaining the hexane fraction by method 2, called RF1 .

Preparation, optimization and characterization of nanoemulsions

Briefly, oleic acid (OA) as oil phase was emulsified in a tween 80 solution as aqueous phase with ultrasonic emulsification (Homogeneizador, OPTIC IVYMEN SYSTEM) with 80% amplitude. The influence of different variables in the preparation of

emulsions were proved. Firstly, different times of sonication (3, 5, 6, 7 and 8 min) were tested with 4g of oleic acid and 96g of a tween 80 solution at 2%w/v. Then, the concentration of tween 80 was changed (1, 1.5, 2, 3 and 4% w/v). Finally, different amounts in oil/aqueous phase were proved (1.5/98.5, 2/98, 3/97, 3.5/96.5 and 4/96 g). All the formulations were prepared in triplicated. The hydrodynamic diameter, polydispersity index (PDI) and zeta potential of each emulsion were measured by dynamic light scattering (Zetasizer Nano ZS90, MALVERN). Zeta potential was determined at 5 different pH (3, 5, 7, 9 and 11). Their stabilities were followed by hydrodynamic diameter, PDI and the separation of oil/aqueous phases over time.

Preparation and characterization of hexane fraction of *Leucophyllum frutescens* and rifampicin loaded emulsions

Different formulations of RF1 and RIF were prepared by ultrasonic emulsification. Briefly, the organic phase containing RF1 or RIF in oleic acid was emulsified in the aqueous phase containing a solution of T-80 (1% w/v). Then, NE were characterized by the measurement of hydrodynamic diameter, polydispersity index (PDI), zeta potential and stability. The actives in NE were quantified by high performance liquid chromatography (HPLC) (WATERS).

RF1 quantification was carried out indirectly, centrifuging the samples (Eppendorf 5415 centrifuge) at 5000 rpm for 30 min, to separate the aqueous phase from the globules. The aqueous phase was taken and analyzed on a Phenomenex C₁₈ column with a flow of 0.2 mL/min at 30 °C. The mobile phase was water (A) and methanol (B) in a isocratic elution 45:55 (A: B) for 60 min. The detection of the RIF peak was at $\lambda=210$ nm.

RIF quantification was carried out indirectly, centrifuging the samples (Eppendorf 5415 centrifuge) at 5000 rpm for 30 min, to separate the aqueous phase from the globules. The aqueous phase was taken and analyzed on a Phenomenex C₁₈ column with a flow of 0.35 mL/min at 30 °C. The mobile phase was water (A) and acetonitrile (B) in an isocratic elution 40:60 (A: B) for 15 min. The detection of the RIF peak was at $\lambda=334$ nm.

Anti-*Mycobacterium tuberculosis* activity of the active fraction of *Leucophyllum frutescens* and rifampicin loaded in the nanoemulsions

The anti-*M. tuberculosis* activity was evaluated on the susceptible strain of *M. tuberculosis* H37Rv by the alamar blue assay in a microplate adapting the methodology used by Molina-Salinas *et al.* (Molina-Salinas *et al.*, 2007). The strain was cultivated in Middlebrock 7H9 broth enriched with OADC (Becton Dickinson and Co., Sparks, MD, USA) at 37 °C for 14 days. After, the strain was adjusted according to the standard scale no. 1 of McFarland and diluted 1:20 for use in the assay. On the other hand, in a 96-well microplate, 200 µL of water was added to the wells in the periphery. While, in the work wells, 100 µL of Middlebrock 7H9 broth enriched with OADC was added. In the first well of each row, 100 µL of each sample (NE blank, NE-RF1 and NE-RIF) and control samples (RF1, RIF and tween 80 solution) were added. Once all the samples were added, serial dilutions 1:2 of each sample were performed, taking 100 µL of the first well and adding them to the second well of the row, then, of the second well, 100 µL was taken to add them to the third, this procedure was repeated until reaching the last well each row; the 100 µL taken from the last well was eliminated. Finally, 100 µL of the bacterial suspension was added to obtain a final volume in all wells of 200 µL. All samples were prepared in duplicate in the same day. The microplates were incubated at 37 °C for 5 days. At day 5, 20 µL of the blue alamar reagent and 12 µL of 10% v/v of tween 80 were added to all the work wells, and the microplates were reincubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was determined from the color change of the blue to pink reagent. All tests were carried out at least in triplicate.

RESULTS AND DISCUSSION

Preparation, optimization and characterization of nanoemulsions

The emulsions were prepared with oleic acid as oil phase and tween 80 as surfactant. Oleic acid was chosen due to *M. tuberculosis* has shown the ability to utilize this fatty acid complexed with triton as a source of carbon for growth was determined (Hedgecock, 1970), and with a nanocarrier based in oleic acid will be possible *M. tuberculosis* takes it more effectively than others.

As part of the optimization, different preparation variables of NE were evaluated. Firstly, the influence of the sonication time (3, 5, 6, 7 and 8 min) on the particle size and PDI was determined (Figure 1). When time was increased the hydrodynamic diameter and PDI decreased from 215.7 ± 3.2 nm to 155.6 ± 0.2 nm and from 0.319 ± 0.025 to 0.244 ± 0.009 , respectively. The ultrasonic emulsification is very efficient in reducing droplet size. Here, the energy is provided through a sonicator probe. It contains piezoelectric quartz crystal which can expand and contract in response to alternating electric voltage. As the tip of sonicator contacts the liquid, it produces mechanical vibration and cavitation occurs. Cavitation is the formation and collapse of vapor cavities in liquid. Thus, ultrasound can be directly used to produce emulsion (Jaiswal *et al.*, 2015). It means, more exposure time to emulsification more energy that allows smaller droplet size. The stability tests showed in the range of 5 to 8 min, a size change around 30 nm from day 0 to day 77 (data not shown), being from 7 min where a homogeneous size distribution was maintained during this period.

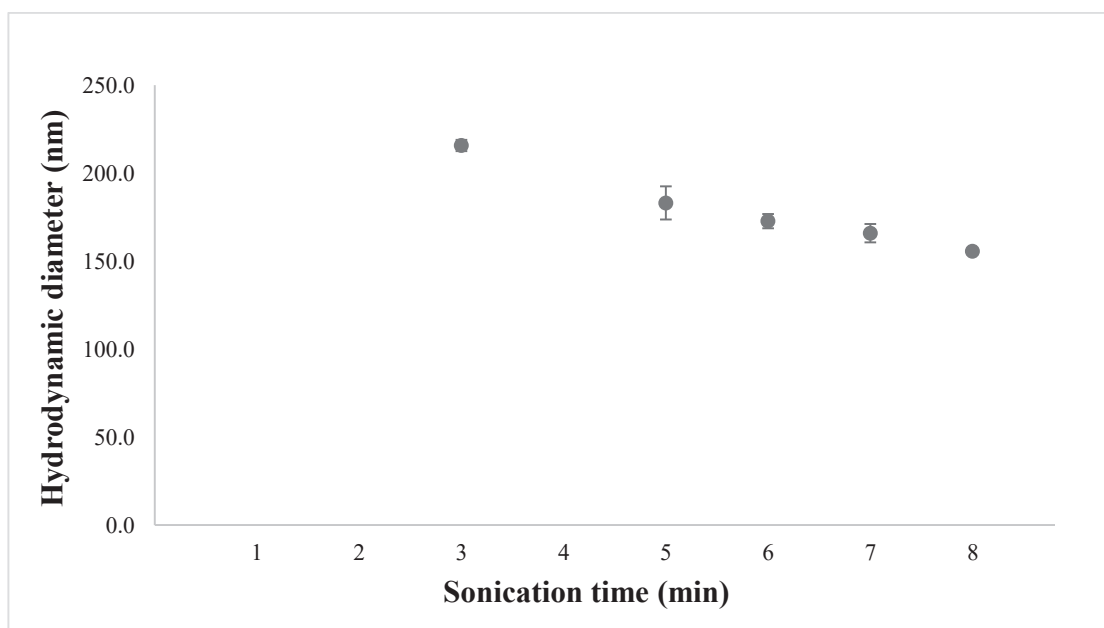


Figure 1. Effect of the sonication time on the hydrodynamic diameter ($\bar{x}\pm\sigma$, n=3).

To continue with the influence of T-80 concentration was used a time sonication of 7 min and 4g of OA. The increased of tween 80 concentration from 1 to 4% w/v

decreased the droplet size from 195.1 ± 1.4 nm to 156.1 ± 4.1 nm but the PDI increased slightly from 0.201 ± 0.006 to 0.285 ± 0.005 (Figure 2). The same behaviors on the droplet sizes were observed in the study carried out by Ghosh *et al.* They prepared NE by ultrasonic emulsification with an oil phase containing cinnamon oil and Tween 80 as surfactant. Three different cinnamon oil and Tween 80 as surfactant ratios (1:1, 1:2 and 1:3 v/v) were proved at 10, 20 and 30 min of ultrasonic emulsification and steady decrease in droplet size of emulsion was observed from ~ 400 to 250 nm, ~ 250 to 96 nm and ~ 200 to 65 nm, respectively. T-80 concentration also played a major role in droplet size of nanoemulsion. Increasing surfactant concentration resulted in decrease in droplet diameter. Formulation with 6% surfactant concentration after sonication for 30 min was found to be 254 nm, whereas formulation with 12% surfactant was 96 nm and formulation with 18% surfactant has lowest droplet diameter of 65 nm after a sonication period of 30 min (Ghosh *et al.*, 2013).

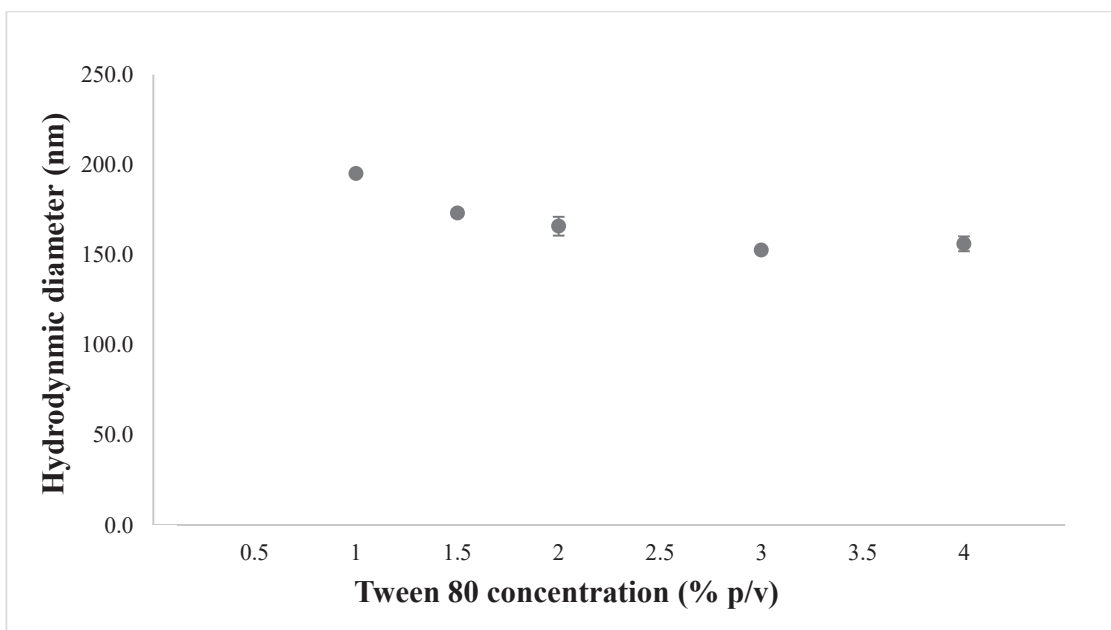


Figure 2. Effect of the concentration of Tween 80 solution on the hydrodynamic diameter ($\bar{x} \pm \sigma$, $n=3$).

Finally, the influence of the oil and aqueous phase amount on hydrodynamic diameter was evaluated, maintaining a sonication time of 7 min and tween 80 solution at 1% w/v. When the oil amount increased while aqueous phase decreased the droplet size at the same time from 126.3 ± 0.7 nm to 195.1 ± 1.4 nm (Figure 3) and the PDI decreased

slightly from 0.259 ± 0.003 to 0.201 ± 0.006 . 0.259 ± 0.003 to 0.201 ± 0.006 . Mantena *et al.* found this influence on size who prepared NE by aqueous phase titration method. Capryol 90 as oil, Tween 20 as surfactant and Transcutol P as cosurfactant. They maintained a constant percentage of surfactant (30%) and co-surfactant (10%) in the formulation and the percentage of oil was 10, 15 and 20% at the same time the percentage of water changed in 50, 45 and 40%. The size increased from 234.9 nm to 285.8 nm (Mantena *et al.*, 2015). The size of the NE droplets supposes an increase, when increasing the oil ratio, the amount of phase is reduced, as a consequence the chains of the surfactant are reduced, increasing the interfacial tension (Du *et al.*, 2016).

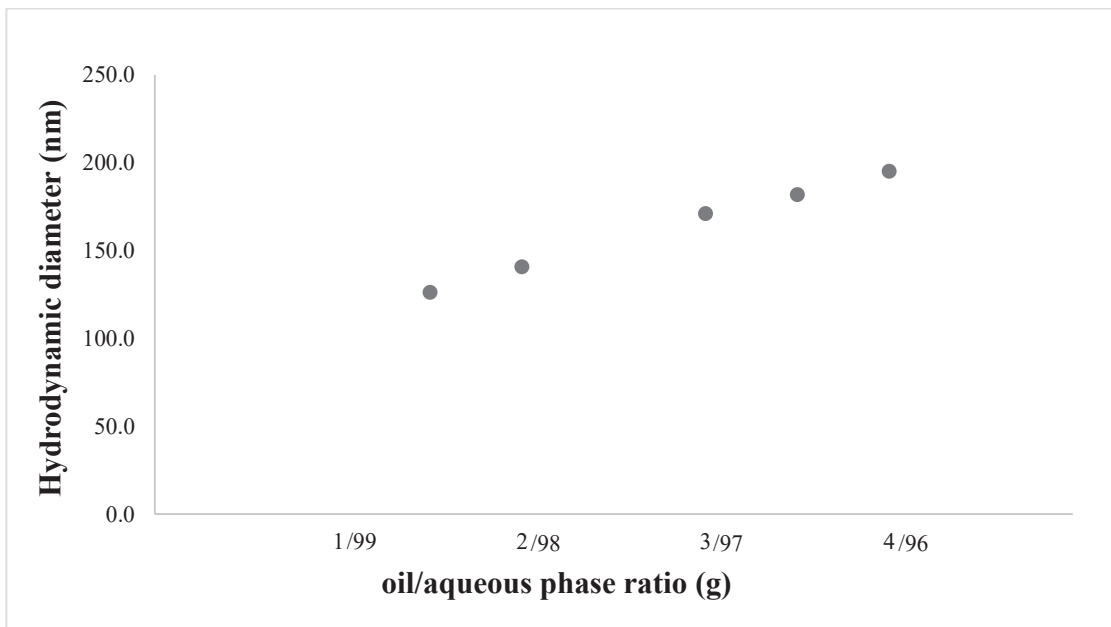


Figure 3. Effect of the ratio on the amount of oleic acid (oil phase) and 1% w/v of Tween 80 solution (aqueous phase) on the hydrodynamic diameter ($\bar{x} \pm \sigma$, n=3).

The NE prepared for the evaluation of the influence of the preparation variables were stable up to 77 days, likewise, all the formulations showed a negative zeta potential. The negative value can be explained by the presence of negatively charged carboxyl groups of oleic acid (Laouini *et al.*, 2012). The negative value can be explained by the presence of negatively charged carboxyl groups of oleic acid (DeRuiter, 2005). The pKa of oleic acid is 5.02 (Pubchem). This explain when we compared at pH 3 the zeta potential is closer 0

(around -3 mV) with pH 5 to pH 11 (-40 to -70 mV) a greatly increased in the negativity can be observed.

Preparation and characterization of the hexanic fraction of *Leucophyllum frutescens* and rifampicin loaded in nanoemulsions

Subsequently, an optimal NE was selected for the preparation of two formulations, for the encapsulation of RF1 and RIF. For the preparation of RF1-NE and RIF-NE the sonication time of 7 min, the concentration of T80 at 1% w/v and the ratio in the amount of oleic acid and aqueous phase of 3.5/96.5 g were selected. The obtained particle size with these variables was around 180 nm (Table I) and a homogeneous size distribution (Figure 4).

Table I. Characterization of nanoemulsions containing rifampicin and the hexane fraction from *Leucophyllum frutescens* roots

<i>Active</i>	<i>Hydrodynamic diameter (nm)</i>	<i>PDI</i>	<i>Encapsulation efficiency (%)</i>	
RIF	179.3±3.5	0.213±0.014	99.9±0.0	
RF1	179.1±1.4	0.217±0.002	<i>Peak 5</i>	75.8±3.5
			<i>Peak 9</i>	86.5±4.7
			<i>Peak 10</i>	72.8±0.4
			<i>Peak 14</i>	70.6±17.4
			<i>Peak 15</i>	84.7±5.3

($\bar{x} \pm \sigma$, n=3)

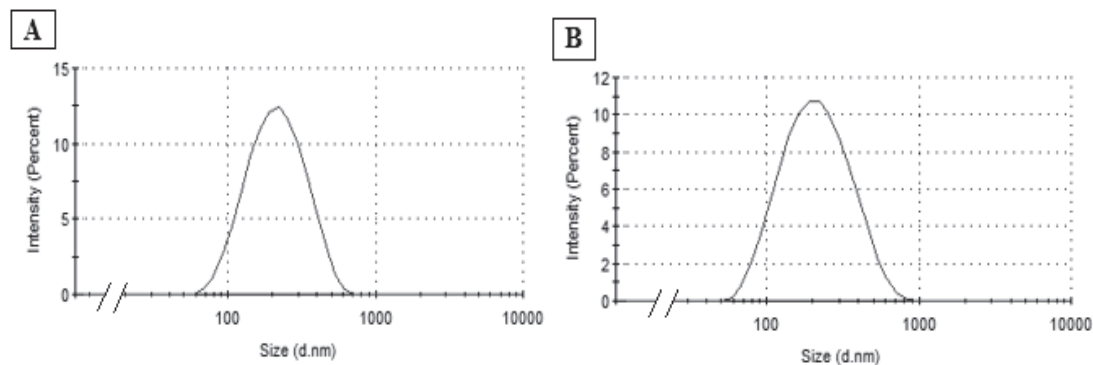


Figure 4. Size distribution of nanoemulsions by intensity percentage: (A) rifampicin loaded nanoemulsion; (B) hexane fraction from roots of *Leucophyllum frutescens*.

Lately, the encapsulation of both active in NE was determined by HPLC. In Figure 5 the chromatogram with the RIF peak in an aqueous solution is observed. When the sample obtained from the centrifugation of the NE was analyzed, it was determined that the molecule is 99% encapsulated. Ahmed *et al.* prepared a NE with RIF using Sefsol 218 as oil and T80 as emulsifier and tween 85 as a co-emulsifier. They found an encapsulation efficiency of around 100% with stability for more than 19 months (Ahmed *et al.*, 2008).

On the other hand, RF1 was dissolved in methanol in order to observe its chromatographic profile, it showed 15 peaks (Figure 6). The peaks 5, 9, 10, 14 and 15 were selected for their quantification within the NE, obtaining an encapsulation efficiency greater than 70%. Similar results were obtained by Ha *et al.* when determined the encapsulation of lycopene in a tomato extract formulated in NE, which was 51 to 65% (Ha *et al.*, 2015). For both formulations, a negative zeta potential (Figure 7) and stability (without phase separation) were observed over 77 days (Figure 8).

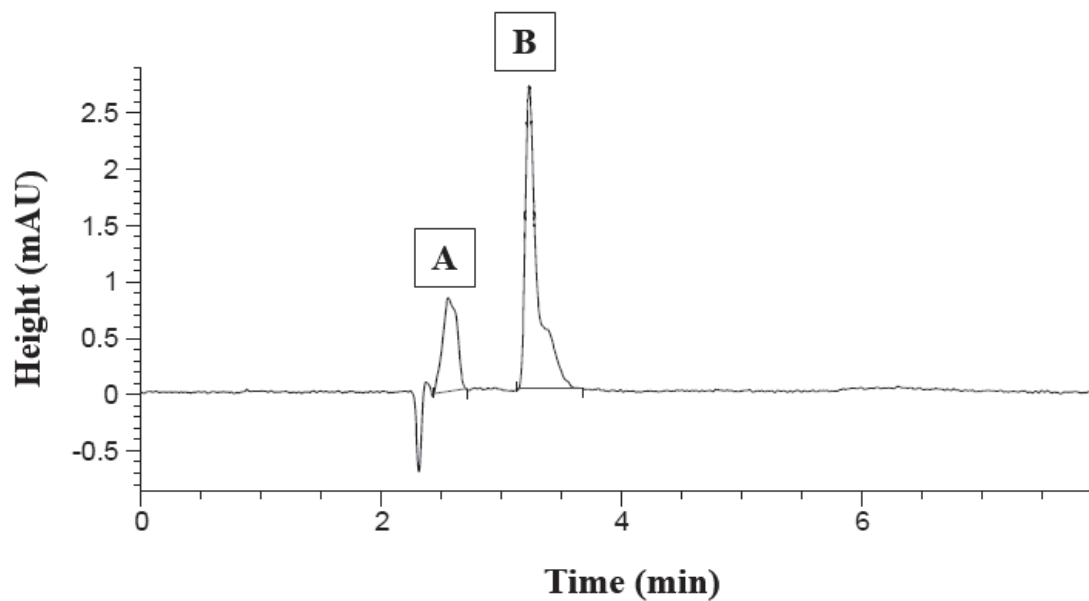


Figure 5. Chromatogram of rifampicin in water (9 µg/mL) obtained by HPLC: (A) water peak; (B) rifampicin peak, retention time = 3.2 min.

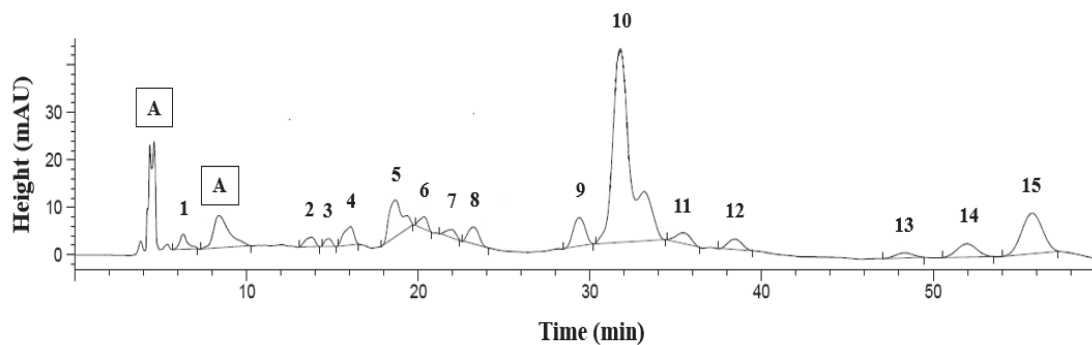


Figure 6. Chromatogram of the hexane fraction from roots of *Leucophyllum frutescens* (230 µg/mL) obtained by HPLC: (A) peaks of methanol.

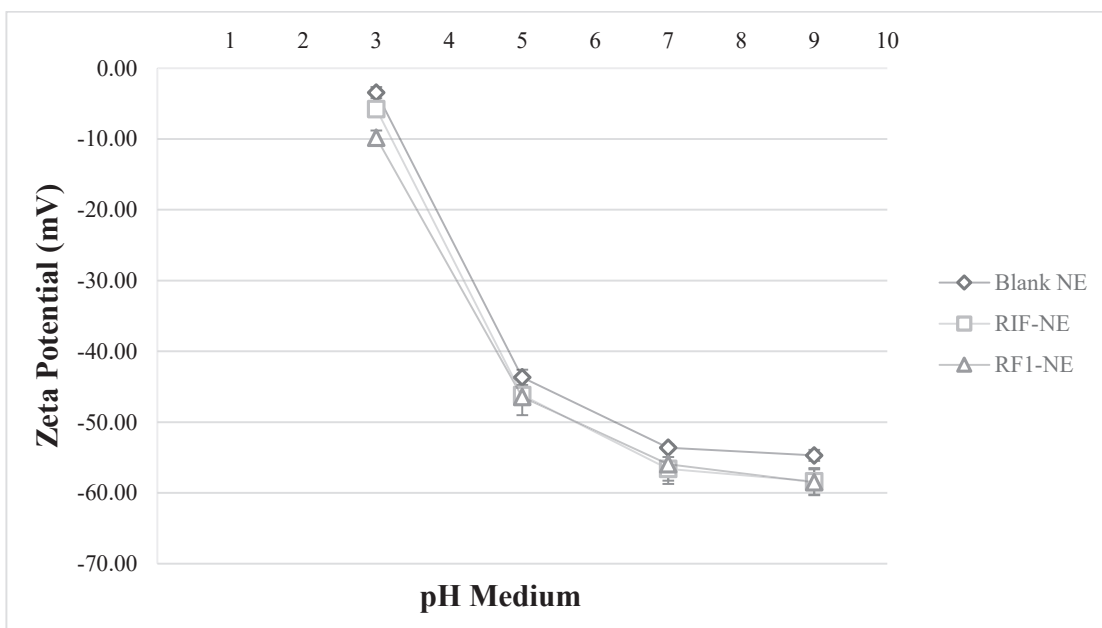


Figure 7. Effect of the pH of the dispersion medium of the nanoemulsions on the zeta potential: blank nanoemulsions (NE), containing rifampicin (RIF-NE) and containing the hexane fraction from roots of *Leucophyllum frutescens* (RF1-NE) ($\bar{x} \pm \sigma$, n=3).

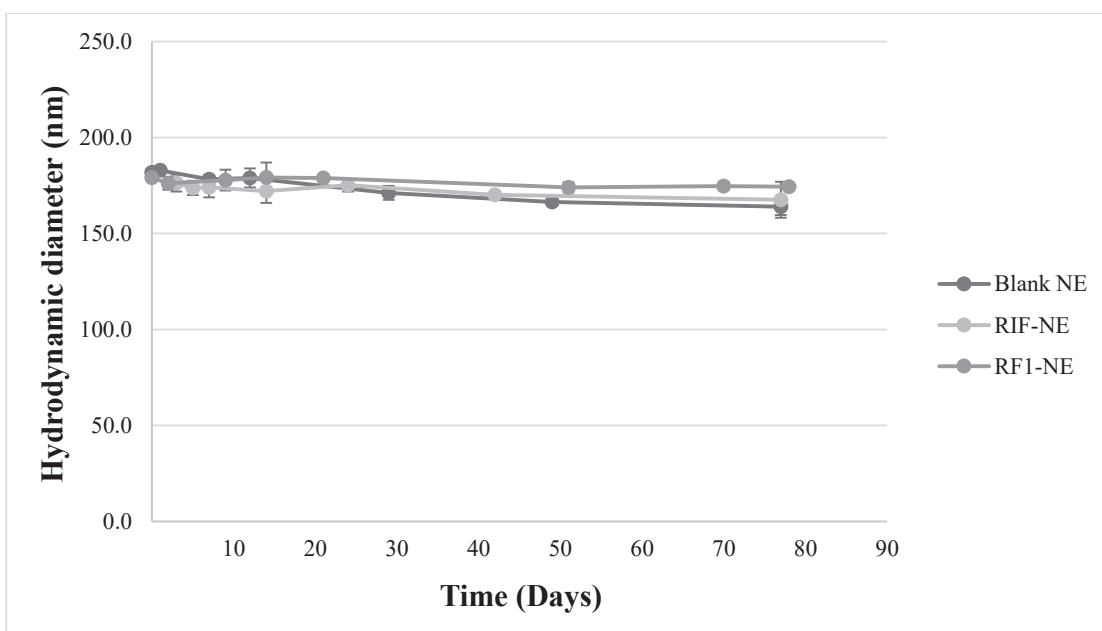


Figure 8. Stability of blank nanoemulsions (NE), containing rifampicin (RIF-NE) and containing the hexane fraction from roots of *Leucophyllum frutescens* (RF1-NE) ($\bar{x} \pm \sigma$, n=3).

Anti-*Mycobacterium tuberculosis* activity of the active fraction of *Leucophyllum frutescens* and rifampicin loaded nanoemulsions

Finally, the activity against *M. tuberculosis* of the three NE (blank NE, RIF-NE and RF1-NE) was evaluated by alamar blue microplate method. The test is based on the color change from blue to pink of the reagent. MIC of RIF and RF1 without encapsulation was 0.19 and 40 µg/mL, respectively. Blank NE showed inhibition when oleic acid and T80 were at 68.10 and 18.75 µg / mL, respectively. While the RIF-NE and RF1-NE showed inhibition when the actives were at a concentration of 0.19 and 0.39 µg/mL, respectively, but OA and T80 were at a concentration of 68.10 and 18.75 µg/mL, respectively, as in blank NE. Choi proved an unsaturated acid called linoleic acid (α and γ form) against *M. tuberculosis*, in both cases fatty acid forms obtained a MIC of 75 µg/mL (Choi, 2016). Therefore, in our study, the inhibition of blank NE can be explained by the presence of oleic acid. It was not possible to observe the effect of the activity of RIF and RF1 when they were in NE due to the presence of the fatty acid.

CONCLUSION

Different parameters were evaluated for the obtaining of NE loaded with RF1 and RIF concluding:

- The optimal parameters for preparation of NE with RF1 and RIF were: sonication time of 7 min, concentration of T80 at 1% w/v and ratio in the amount of oleic acid and aqueous phase of 3.5/96.5 g, having a diameter around 180 nm, homogenous size distribution, negatively charged and, high encapsulation efficiency.
- The stability of NP was for 77 days.
- Both formulations showed high efficiency of encapsulation.
- The anti-*M. tuberculosis* activity showed inhibition in the same concentrations including blank NE, this behavior can be attributed due to the presence of oleic acid as oil phase.
- The formulations of NE with RF1 is promising as a co-adjuvant for the Tb treatment.

VIII. GENERAL CONCLUSIONS

In this study some investigations with the extracts and fractions from *L. frutescens* were carried out in order to obtain a broader knowledge about the biological activity present in the plant. Likewise, the vegetable samples that showed activity were encapsulated in free systems of organic solvents. NP has shown to be a good alternative to load actives into nanoparticles. As good delivery systems, actives loaded into nanoparticles show stability, protection, controlled release and targeted. Different parameters during nanoprecipitation process can be modified to obtain a formulation with the desirable characteristics on the size, storage stability, active encapsulation and electrostatic charges.

Based on the antimycobacterial activity revealed by previous studies, the first part of this work was focused on testing the effect of *L. frutescens* against *M. tuberculosis* to propose it as a co-adjuvant of the drugs used for the tuberculosis treatment, such as RIF. To obtain the methanol extract from leaves and roots of *L. frutescens*, an ultrasound method was developed, which allowed a good performance to be obtained in a short time (2 h). Two different fractionation methods were developed in order to obtain the hexane fraction of leaves and roots, the percentage of yield obtained with both methods was close. However, method 2 was selected to continue with the following partitions because it is faster and easier to execute. The chromatographic profiles of the extracts and fractions were obtained through HPLC, these results reveal that there is the same number of peaks between the extract (leaves or roots) and the fractions obtained from it; in roots there is higher number of peaks than leaves.

The evaluation of the activity against *M. tuberculosis* of the extracts and fractions of *L. frutescens* revealed that in roots are more activity than in leaves, specifically, EMR, FHR and RF1 (MIC = 100, 40 and 40 $\mu\text{g}/\text{mL}$, respectively). Biodegradable NP of RIF and vegetable samples (EMR, FHR and RF1) of *L. frutescens* were prepared by nanoprecipitation. Formulations of NP showed a size from 140 to 190 nm, with homogeneous distributions and negative zeta potential. RIF had a MIC of 0.20 $\mu\text{g}/\text{mL}$, but when RIF is encapsulated in NP-PLGA-PVAL, it enhanced its activity (0.10 $\mu\text{g}/\text{mL}$) against *M. tuberculosis*. On the other hand, the hexane fractions in NP had more activity

than free. The most active formulation was NP-PLGA-FHR and NP-PLGA-RF1 with a MIC of 80 µg/mL for both.

The combination of FHR and RF1 with RIF decreases the MIC value in both cases, demonstrating a better effect. Finally, the toxicity of the best samples was proved, at the active concentrations against *M. tuberculosis* RIF and NP-RIF did not present hemolysis. Whereas, RF1 and NP-RF1 showed hemolysis. However, when RF1 is combined with RIF, the concentrations decreased from 40 to 10 µg/mL for RF1 and from 80 to 20 µg/mL for RF1 loaded NP, and at these concentrations there is no presence of hemolysis. This suggested that the encapsulation of RF1 in NP favored the reduction of its toxicity. Therefore, an alternative to make use of the hexane fraction from roots of *L. frutescens* with potential activity against *M. tuberculosis*, at concentrations below the hemolytic effect, is to combine it with conventional antituberculosis drugs such as RIF, and thus, to enhance the activity.

Because of *L. frutescens* has been less studied for its antioxidant capacity, the second part of this work was focused on the antioxidant effect of the previously obtained extracts and fractions. The extracts and fractions of the *L. frutescens* plant showed a certain degree of protection, avoiding the lysis of RBC caused by the induced oxidation of AAPH reagent. FHH, EMR, FHR, RF1, RF2 and RF4 presented their maximum antioxidant activity at low concentrations and, subsequently, decreased when they were tested at higher concentrations due to a hemolysis effect. The EMH, HF1, HF2, HF3, HF4, HF5 and RF5 were samples with antioxidant activity from 25 to 200 µg/mL and without hemolytic effect, being selected the fraction HF4 for the studies that proceeded. Suspensions of HF4 loaded NP were obtained by the nanoprecipitation technique, NP had a particle size around 200 nm with a homogeneous distribution. A chromatographic method was partially validated to be used for the quantification of the peaks-components present in HF4, from which the encapsulation of four peaks in NP was determined, it revealed a favorable incorporation of the *L. frutescens* fraction. Finally, when HF4 was incorporated in NP the antioxidant activity was conserved. For this reason, the incorporation of HF4 loaded NP is promising as an antioxidant agent because the

encapsulation of the fraction is achieved, its properties are preserved, and it is non-toxic at the active concentrations.

An important part of the characterization of nanoparticulate systems is the determination of the percentage drug loading and encapsulation efficiency, for this reason a method of quantification by HPLC was developed and validated using the peaks-components of the plant as internal markers. Therefore, in this work we proposed a new strategy for the quantification of extracts or fractions of *L. frutescens*.

In the last part of the study, to make use of a sustained release system different of NP was proposed. Studies with NE were carried out testing the effect of sonication time, surfactant concentration and oil/water ratio on particle size, PDI, zeta potential and stability. The optimal formulation was selected for the encapsulation of RIF and the active fraction RF1 against *M. tuberculosis*. NE had a diameter around 180 nm, homogeneous size distribution, negatively charged and stable for 77 days. Both formulations showed high encapsulation efficiency. The anti-*M. tuberculosis* activity showed inhibition at the same concentrations including blank NE, this behavior can be attributed to the presence of oleic acid as oil phase. Thus, the optimal characterized and tested NE can be promising in the inhibition of *M. tuberculosis*.

With the results obtained in our work, the knowledge about the *L. frutescens* plant is extended, either by its activity against *M. tuberculosis* or by its antioxidant activity. Likewise, it is revealed that NP or NE are a promising alternative to load extracts or fractions of the plant, converting them into a viable formulation for its administration.

IX. PERSPECTIVES

Based on the results and conclusions obtained in this Doctoral Thesis, the perspectives are oriented in different directions.

In the Chapter 1, the results related to RIF, *L. frutescens*, NP and its activity against *M. tuberculosis* were shown. For this first section, future research would be about *in vitro* release studies of the active formulations obtained in order to know the time of release of the actives to the environment in which they would be in the body and, also, to propose their release mechanisms. Because the target organ of the formulations are macrophages, *in vitro* studies on macrophages would be promising. Likewise, from the favorable results obtained on the combination of the drug RIF and the formulation of NP-PLGA-RF1, we propose to work with the tests to determine the synergism effect. On the other hand, it would be interesting to know about the distribution of the active formulations in an organism, for which we suggest to carry out *in vivo* biodistribution studies of RIF and RF1 free and encapsulated into NP, individually and in combination. The final perspective to this chapter is to carry out the studies of isolation and identification of the compounds from the extracts and root fractions of *L. frutescens* that have the best activity against *M. tuberculosis*, encapsulation of the compounds in NP, the characterization of NP and studies of their effect on *M. tuberculosis*.

In the Chapter 2, the results related to *L. frutescens*, NP and its antioxidant activity were presented, due to the promising results obtained with the antioxidant effect of the plant, future investigations would be studies of *in vitro* release of the active encapsulated into formulations in order to know the time of release towards the environment in which they would be in the organism, also, to propose its release mechanisms and *in vivo* biodistribution studies.

Finally, in the Chapter 3, the results related to RIF, RF1, NE and their activity against *M. tuberculosis* were shown. Based on our research, it will be of great interest to

work on the formulation, determining the oil phase and aqueous phase that does not present activity in the microbiological studies.

For all the formulations obtained in this thesis work, other *in vitro* and *in vivo* methods should be carried out to expand knowledge about toxicity.

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APPENDIX 1

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Review

Nanoprecipitation process: From encapsulation to drug delivery



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ABSTRACT

Drugs encapsulation is a suitable strategy in order to cope with the limitations of conventional dosage forms such as unsuitable bioavailability, stability, taste, and odor. Nanoprecipitation technique has been used in the pharmaceutical and agricultural research as clean alternative for other drug carrier formulations. This technique is based on precipitation mechanism. Polymer precipitation occurs after the addition of a non-solvent to a polymer solution in four steps mechanism: supersaturation, nucleation, growth by condensation, and growth by coagulation that leads to the formation of polymer nanoparticles or aggregates. The scale-up of laboratory-based nanoprecipitation method shows a good reproducibility. In addition, flash nanoprecipitation is a good strategy for industrial scale production of nanoparticles. Nanoprecipitation is usually used for encapsulation of hydrophobic or hydrophilic compounds. Nanoprecipitation was also shown to be a good alternative for the encapsulation of natural compounds. As a whole, process and formulation related parameters in nanoprecipitation technique have critical effect on nanoparticles characteristics. Biodegradable or non-biodegradable polymers have been used for the preparation of nanoparticles intended to *in vivo* studies. Literature studies have demonstrated the biodistribution of the active loaded nanoparticles in different organs after administration *via* various routes. In general, *in vitro* drug release from nanoparticles prepared by nanoprecipitation includes two phases: a first phase of "burst release" which is followed by a second phase of prolonged release. Moreover, many encapsulated active molecules have been commercialized in the pharmaceutical market.

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Contents

1. Introduction	67
2. Encapsulation of active pharmaceutical ingredients	67
3. Encapsulation based marketed products	68
4. Nanoprecipitation	68
5. Precipitation mechanism	71
6. <i>In vitro</i> release profile	72
7. Applications	73
7.1. Medicine	73
7.1.1. Synthetic compounds encapsulated in polymeric particles	73
7.1.2. Natural compounds encapsulated in polymeric particles	73
7.1.3. Protein based particles	75

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Research Article

Quantitative Aspect of *Leucophyllum frutescens* Fraction before and after Encapsulation in Polymeric Nanoparticles

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The interest on plants has been focalized due to their biological activities. Extracts or fractions from plants in biodegradable polymeric nanoparticles (NP) provide many advantages on application studies. The encapsulation of the extract or fraction in NP is determined for the establishment of the test dose. HPLC method is an alternative to calculate this parameter. An analytical method based on HPLC for quantification of a hexane fraction from *L. frutescens* was developed and validated according to ICH. Different concentrations of the hexane fraction from leaves (HFL) were prepared (100–600 µg/mL). Linearity, limit of detection, limit of quantification, and intra- and interday precision parameters were determined. HFL was encapsulated by nanoprecipitation technique and analyzed by HPLC for quantitative aspect. The method was linear and precise for the quantification of the HFL components. NP size was 190 nm with homogeneous size distribution. Through validation method, it was determined that the encapsulation of components (1), (2), (3), and (4) was 44, 74, 86, and 97%, respectively. A simple, repeatable, and reproducible methodology was developed for the propose of quantifying the components of a vegetable material loaded in NP, using as a model the hexane fraction of *L. frutescens* leaves.

1. Introduction

Since prehistoric times, medicinal plants have been used as treatments for numerous human diseases [1, 2]. Recently, research articles have proved biological activities of some plant extracts as antioxidant [3, 4], anti-inflammatory [4], antimicrobial [5, 6], and anticancer [7] activity. Organic solvents are used in the extraction process to obtain the active plant material [8] and as carrier in the assays for extract application. However, for *in vivo* administration, an organic solvent would result in toxicity for the organism [9].

Different carriers as nanocomposite films [10], microparticles [11], and nanoparticles [12, 13] have been a good alternative as drug delivery systems. Particularly, polymeric

nanoparticles (NP) are promising as carriers which present sustained release and protection to the active [14] and targeting to specific organs [15, 16]. During NP preparation the organic solvent has to be eliminated. Researches in natural products encapsulation have demonstrated beneficial effects. *Arrabidaea chica* is a plant with healing properties employed in folk medicine for wound healing, inflammation, and gastrointestinal colic. Servat-Medina et al. studied the antiulcerogenic activity. *A. chica* hydroalcoholic extract was incorporated in chitosan-sodium tripolyphosphate NP. An *in vitro* study in human skin fibroblasts showed biocompatibility. *In vivo* study proved that *A. chica* hydroalcoholic extract-loaded NP enhances its antiulcerogenic activity [17]. Similar behaviors were obtained by Kwon et al. They investigated

APPENDIX II
PARTICIPATION IN CONGRESSES

“Cuantificación de rifampicina incorporada en nanopartículas poliméricas por HPLC para su potencial aplicación biológica”. XXIX Congreso Nacional de Química Analítica y XIX Simposio Estudiantil. Villahermosa, Tabasco, Mexico. June-July 2016. Poster.

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“Caracterización de extractos de *Leucophyllum frutescens* (cenizo) obtenidos mediante dos métodos de extracción”. 10^a. Reunión Internacional en Investigación de Productos Naturales. Yucatán, Mexico. May 2014. Poster.