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"Development of a mucoadhesive buccal film as a carrier of *Lippia graveolens* oil emulsions and its *in vitro* evaluation against *Candida albicans*"

TESIS

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Dedication

To my mother, Rosa Amelia Rivera Ulloa, for trusting me and letting me take this path, even though it meant apart ourselves in distance. Your extreme effort, dedication and organization in everything you do, served me as a guide to achieve what seven years ago I set myself as an objective.

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Abstract

The purpose of this work is to develop a mucoadhesive film that serves as a carrier for *Lippia graveolens* essential oil (LGO) emulsion and to evaluate *in vitro* their inhibitory effect against opportunistic pathogen *Candida. albicans*. For this, different ratio Chitosan: Pectin (100:0, 75:25, 50:50, 25:75, 0:100) films were obtained by casting technique with different concentration of glycerol as a plasticizer (0, 10 and 30%). Water-uptake, erosion, and mucoadhesion were evaluated.

Films with CH:P ratio of 100:0 with 30% glycerol, 75:25 with 10% glycerol and 50:50 with 10% glycerol showed better mucoadhesion and physical properties. Characterization of LGO was carried out, and major components thymol and carvacrol were quantified by GC-MS, these phenols were present in a 2:1 ratio. Three different emulsions with LGO were developed and characterized, their inhibitory effect was evaluated against *C. albicans*. Arabic gum (GA), Hydroxylated lecithin (LecH) and polysorbate 80 (T80) were used as emulsifiers. The particle size of emulsions was 1597±11.1, 179±5.5 and 137.3±4.2 respectively.

All emulsions had an inhibitory effect against *C. albicans*. Volumes of emulsions that inhibit the 99% of *C. albicans* were used for the *in vitro* test of films together with emulsion.

With this work it was possible to develop three different emulsions with an inhibitory effect against *C. albicans*, likewise, these emulsions showed an inhibitory effect in a localized way when evaluated together with the mucoadhesive films against *C. albicans*.

I. Introduction

I. 1 Oral cavity and related disorders

The oral cavity is important not only because is the route by which we ingest the nutrients that we need, but also plays an important role in the body's immune system since most of the time is exposed to a variety of antigens and pathogens (Ormond *et al*., 2015). Based on the region of the oral cavity, distinct patterns of maturation in the epithelium of the human oral mucous are found, however, three distinct layers of the oral mucous are distinguished (Chinna *et al*., 2011):

1. **Epithelium**
    1. Non-keratinized (soft palate, ventral Surface of the tongue, lips, cheeks, floor of the mouth)
    2. Keratinized (hard palate, non-flexible regions)
2. **Basement membrane**
3. **Connective tissue**

Good oral health is a goal of modern dental care; nevertheless, this cannot always be maintained since malignant diseases of the mucous are increasingly common (Rautemaa *et al*., 2007). Over 400 different types of oral cavity disorders exist, such as oral mucositis, gingivitis, periodontitis, dental caries, oral infections and lesions which are usually treated by local therapy (Sao *et al*., 2009). Some oral pathogens have been associated with periodontal diseases, but also oral infections can be triggered by oral mucosal lesions (Rautemaa *et al*., 2007).

I.1.2 Buccal candidiasis

Buccal candidiasis is the most common fungal infection in the oral cavity, is caused by the overgrowth of commensal *Candida* species, being the most common *C. albicans* who is considered an opportunistic pathogen (Costa, *et.al*.2014). This infection is triggered by systemic or local factors. The systemic factors are favored by endocrine disorders, immune defects, physiological (age) and nutritional factors (vitamin B12 deficiency) (Otero *et al*., 2015; Tarçın, 2011). Local factors can be xerostomia, medications (antibiotics, corticosteroids), high-carbohydrate diet, denture stomatitis, angular cheilitis, hairy tongue, and smoking, among others (Otero *et al*., 2015; Tarçın, 2011).

I.1.3 Drugs used for buccal candidiasis

The most common medications for the treatment of *Candida* in the oral cavity are Miconazole, Clotrimazole, Ketoconazole, and Fluconazole (Singh, *et.al* 2014). The azoles derivatives are generally targeted to the ergosterol biosynthetic enzyme lanosterol 14α demethylase (Ahmad *et al*., 2011). The ergosterol is a sterol present in the cell membrane of fungi, it is responsible for maintaining membrane integrity in fungal cells, thus inhibition of the lanosterol 14α demethylase enzyme leads to depletion of ergosterol and accumulation of sterol precursors, consequently the structure and function of the plasma membrane is altered (**Fig. 1**) (Ahmad *et al*., 2011; Ghannoum and Rice, 1999; Sanglard *et al*., 2003). MIC range for these drugs is around 0.03-16 µL, but, if *C. albicans* develops resistance, MIC tends to increment.

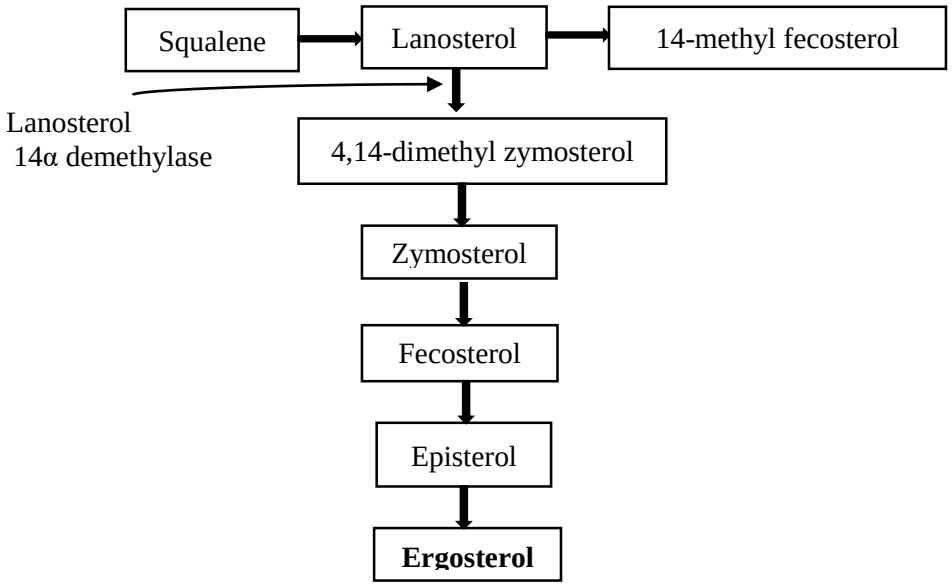


Fig. 1. Simplified schematic representation of ergosterol biosynthetic pathway in *Candida albicans* (Ghannoum and Rice, 1999; Sanglard et al., 2003).

Despite of the existing drugs for the treatment of oral candidiasis, there is a particular interest in developing new alternatives to combat *Candida spp.*, since it has been reported an increased resistance of *Candida* to some antifungal drugs (Manzano *et al*., 2008; Rodrigues *et al*., 2010; Sanglard *et al*., 2003; Spampinato and Leonardi, 2013; Warnock, 1992; Whaley *et al*., 2017). In addition, there are reports from the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) indicating that ketoconazole tablets can cause severe liver damage, adrenal gland problems and harmful interactions with other drugs (FDA, 2013; EMA 2013).

I.1.4 Natural treatments for *Candida albicans*

Nowadays, there is an increasing interest in plant extracts for medicinal uses, rather than drugs for local treatment. Some of the advantages of using natural products are wide acceptance and better tolerance for the patient, fewer adverse effects, and in the case of bacterial or fungal infections, can be an alternative to drug resistance (Yap *et al*., 2014). The strong antifungal activity of some major compounds of essential oils has been reported. The efficiency of carvacrol, geraniol, and thymol, for treatment of candidiasis, has been demonstrated. Other compounds extracted from the plants with anti mycological activity are saponins, alkaloids, peptides, and proteins (Spampinato and Leonardi, 2013).

Essential oils (EOs) are mixtures of non-volatile and volatile, lipophilic compounds, which can be classified in alkaloids, flavonoids, isoflavones, monoterpenes, phenolic acids, carotenoids, and aldehydes. In the last ten years, there has been a considerable increase in the number of publications related to EOs, due to their multiple properties (antioxidant, antiseptic, anti-inflammatory, antibacterial, antifungal, analgesic) (Donsì and Ferrari, 2016). Besides the eco-friendly and biodegradable nature, the synergistic effect of compounds in the essential oil increments its activity in comparison to the individual substances (Sao *et al*., 2009). **Table 1** shows some of the EOs that have been used against different *Candida spp.*

Table 1. Essential oils with anti-candida activity

Essential oil	Bioactive	Efficiency	Author
a) Thymus capitatus ***b) Mentha spicata*** ***c) Satureja montana***	a) Thymol b) Carvone c) Carvacrol	a) MIC 0.002-1% v/v b) MIC 2-4% v/v c) MIC 0.002-1% v/v	(Bona *et al*., 2016)
Cymbopogon citratus	Citral	Partial inhibition with 8 µL	(Bona *et al*., 2008)
***LGO* (Guatemala)**	Thymol , carvacrol, p-cymene	MIC 0.77µL/mL	(Salgueiro *et al*., 2003)
Lippia sidoides	Thymol, carvacrol	MIC 2.5mg/mL	(Botelho *et al*., 2007)
Origanum vulgare	Thymol, carvacrol	MIC 1.2-5 µL/mL MFC 1.2-10 µL/mL	(Cleff *et al.*, 2010)
-	a) Thymol b) Carvacrol	a) MIC 75-90mg/mL b) MIC 100-125mg/mL	(Ahmad *et al.*, 2011)
-	Thymol	MIC 39-78 µg/mL MFC 39-78 µg/mL	(Dias *et al*., 2015)
Cinnamomum zeylanicum	-	MIC 312.5-625 µg/mL MFC 625-22500 µg/mL	(Castro and Lima, 2013)

I.2 Essential oil of Mexican *Lippia graveolens* plant

Oregano is an herbaceous, perennial and aromatic plant (**Fig. 2)**. Taxonomically, it is represented in four families: Asteraceae, Fabaceae, Lamiaceae, and Verbenaceae. Some of the species with the highest economic importance are *Origanum vulgare, Origanum onites,* and *Lippia graveolens*. The last one is endemic from Mexico, the second world's largest oregano producer country. The most important production belongs to the states of Chihuahua, Durango, Tamaulipas, Coahuila, Jalisco, Zacatecas, Queretaro, Hidalgo and Baja California Sur (García *et al*., 2012). Uses reported for Mexican oregano are varied, it is commonly used as a condiment for food. Also, the pharmaceutical (analgesic, bactericide, fungistatic, antipyretic, antidiarrheal) and perfume industry have taken advantage of its essential oil (Pérez *et al*., 2011).

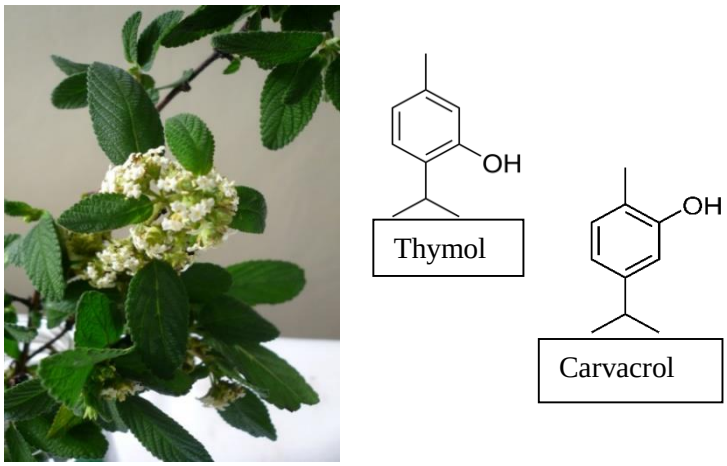


Fig. 2 *Lippia graveolens* plant and the major components of its essential oil

For Mexico, is of great socioeconomic relevance. Annually, 6500 tons of dry leaves are used, leaving an economic income superior to 160 million of pesos for exporting 90% of this. Furthermore, recollection of oregano leaves is realized by the peasants from the most marginal communities, what represents an alternative option for their economic support (Pérez *et al*., 2011).

Different institutions of education and research in the country are making an effort to diversify the use of oregano products, like its essential oil. EO from oregano plant can be extracted mainly by hydrodistillation (Botelho *et al*., 2007; Castillo *et al*., 2007; Hernández *et al*., 2014; Salgueiro *et al*., 2003; Tongnuanchan and Benjakul, 2014).

Oregano essential oil has been shown to possess the highest antimicrobial activity compared to other essential oils, due to its major components thymol and carvacrol (Alvarez *et al*., 2014). These components can serve as quality indicators, where 45-50% content of both phenols is considered good quality for *LGO* (Castillo *et al*., 2007). Variations in the chemical composition may depend on environmental factors like the geographic source, the storing conditions, the method of extraction and the harvesting season (Arana *et al*., 2010; Castillo *et al*., 2007).

Antifungal and antibacterial activities of LGO have been reported previously, mainly against *A. alternate, C. albicans, E. coli, Ps. aeruginosa, Staph. aureus,* and *Salmonella* (Alvarez *et al*., 2014; Arana *et al*., 2010; Córsico *et al*., 2014; Nostro *et al*., 2017).

The high reactivity and low water solubility (hydrophobicity) have limited their application since it is a challenge their direct incorporation into different matrices (Donsì and Ferrari, 2016). For that, simple solutions, colloids, emulsions, polymeric films, and powders among others, have been developed to encapsulate lipophilic bioactive (I.3 Delivery Systems).

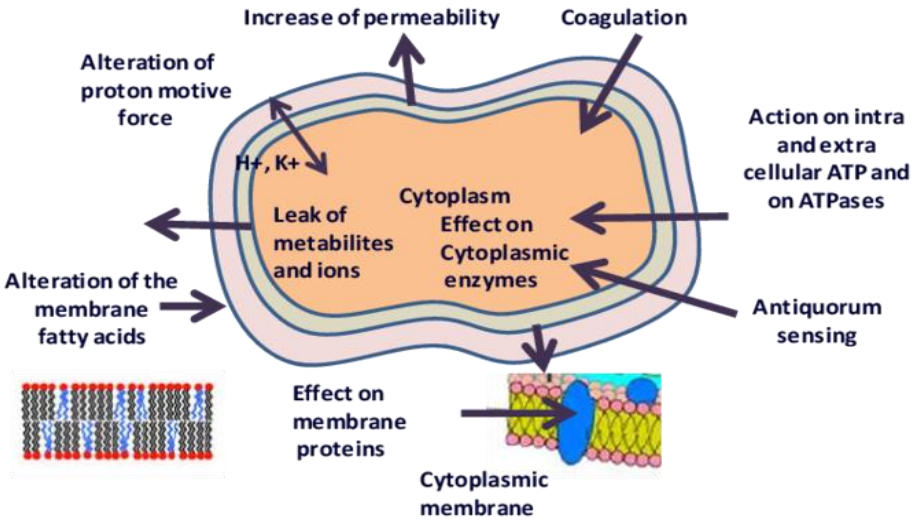


Fig. 3 Proposed mechanisms of essential oils to inhibit microbes (Retrieved from (Nazzaro *et al*., 2013)

EOs can affect both the external envelope of the cell and the cytoplasm. The membrane of pathogen microorganisms is the main target of EOs, because of their hydrophobic nature, EOs can penetrate the microbial cells and trigger a series of mechanisms like degradation of the cell wall, damage to the cytoplasmic membrane and cytoplasm coagulation. Also, the increase in the wall permeability leads to the leakage of the cell content, reducing the intracellular ATP pool and destroying the electron transport system. The sustained loss of ions or metabolites can compromise the microbial metabolism and lead to cell death.

The different molecules present in EOs are responsible for the multiple antimicrobial mechanisms, hence, the inhibitory effect of EOs cannot be attributed to a single mechanism but to the consequence of the multiple activities between molecules **Fig. 3.**

I.3 Delivery Systems

Different delivery systems for oral mucous have been developed and include solutions, suspensions, gels, creams, ointments, tablets, films, wafers, patches, microparticles, and sprays, among others **Fig. 4**(Patel *et al*., 2012) Buccal tablets and films are preferred for local administration, because they have better retention time, unlike liquids and semisolids dosage forms (Hearnden *et al*., 2012).

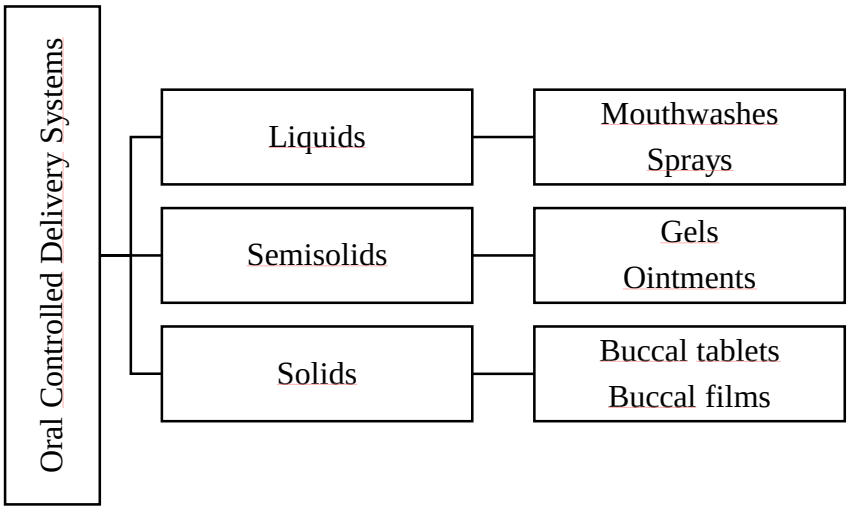


Fig. 4. Classification of oral delivery systems based on the physic state of the dosage form

1.3.1 Liquids

Liquid dosage forms consist of one or more ingredients in a liquid vehicle. These dosage forms can be divided into two major categories: solutions (the active ingredients are dissolved in the liquid vehicle) and dispersions (undissolved ingredients are dispersed throughout a liquid vehicle). Some examples of liquid dosage forms are mouthwashes, sprays, and emulsions). Some disadvantage of these traditional systems is poor retention in the oral cavity leading to a suboptimal therapeutic effect (Nguyen and Hiorth, 2015).

1.3.2 Semisolids

Creams, gels, and ointments are some examples of semisolid dosage forms. They have the advantage of easy dispersion throughout the oral mucous. Nevertheless, as liquid dosage forms have a poor retention time of the bioactive at the site of application (Salamat *et al*., 2005).

1.3.3 Solids

Examples of solid formulations are tables and lozenges. These dosage forms get dissolved into the oral cavity and improve the retention time of the bioactive in a local area. A drawback of tablets is their lack of physical stability, leading to poor patient compliance.

Buccal films are other dosage forms and are preferred over buccal tablets in terms of flexibility and comfort (Salamat *et al*., 2005).

Buccal films

When an ideal buccal film is designed, three properties are important to consider: 1) Mucoadhesion (maintain its position in the buccal mucous for a few hours), 2) Controlled release of the drug (can be achieved with the polymer network and its degree of solubility and swelling) 3) Unidirectional release of the drug (a backing layer made with a water impermeable material can be added to the adhesive polymer film) (Remuñán *et al*., 1998). In addition, it shall be flexible, elastic, soft and resistant to mouth movements. Water-uptake of films, shall not be too extensive in order to prevent discomfort, moreover, mucoadhesion is essential for the purpose of these systems (Salamat *et al*., 2005).

1) Mucoadhesion

The mucoadhesion is the adhesion between two materials, where at least one of them has a mucosal surface. In this mechanism chemical interactions occur (Van der Waals, hydrogen bonding, electrostatic, hydrophobic) as well as physical interactions (diffusion) (Raghavendra Rao *et al.*, 2013; Salamat *et al*., 2005). This property can be achieved by selecting an adhesive polymer.

There are different environmental and polymer-related factors affecting the mucoadhesion (**Fig. 5**). In general, it has been reported that bioadhesive strength of a polymer increases with high molecular weight (>100,000 Da) and with a positive charge, especially in a neutral or alkaline medium. As one mechanism of mucoadhesion is the diffusion, it is important that the polymer chains contain some degree of flexibility in order to obtain entanglement with the mucous. Also, the presence of functional groups that are able to form hydrogen bonds is favorable for the mucoadhesion. The microenvironment surrounding the buccal film also affects the mucoadhesion. The pH of the oral cavity (5.5-7.0) can alter the ionization state of the adhesive polymer and some enzymes can degrade it (Chinna *et al*., 2011).

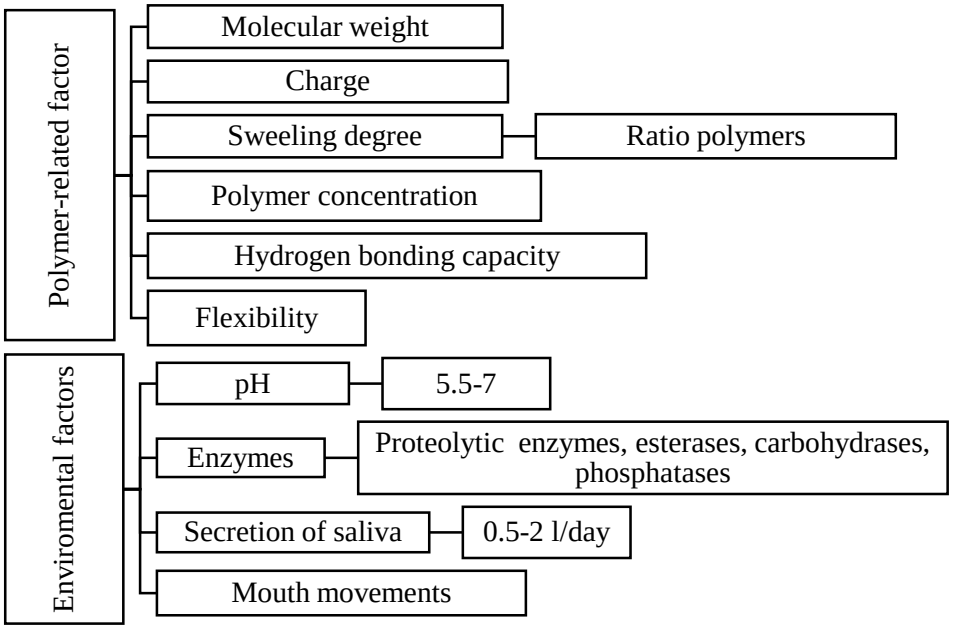


Fig. 5. Factors affecting the mucoadhesion of the films

I.3.2 Components of buccal films

a) Mucoadhesive polymers

Some characteristics to consider when selecting the polymer are biocompatibility, fast adhesion to moist surfaces, availability in the market, and easy incorporation of the drug into the formulation. (Andrews *et al*., 2009; Raghavendra Rao *et al*., 2013). Mucoadhesive polymers can be classified by source, solubility in water and charge. (Raghavendra Rao *et al*., 2013). Being the charge, the property most relevant for mucoadhesion. Since electrostatic interactions can occur between opposites charge, one of the mucous surface and the other from the polymer. Ionic and cationic polymers have exhibit the greatest mucoadhesive strength (Andrews *et al*., 2009). Chitosan is a cationic, non-toxic and biodegradable polymer, composed of N-acetyl-glucosamine units. This polymer is obtained by the alkaline deacetylation of chitin (the principal component of the exoskeleton of crustaceans and cell walls of fungi) and is considered one of the most promising polymers in wound dressing (Hurler and Škalko-Basnet, 2012). Is extensively used in the pharmaceutical, food, and clinical context. The cationic character of chitosan derives from free amino groups, which allows the interaction with many anionic polymers like pectin (Sao *et al.*, 2009). Pectin is a natural, non-toxic and anionic polymer containing D-galacturonic acid units, is extracted from cell walls of most plants and citrus fruits. Depending on the methylation degree of its carboxyl groups, can be categorized into high (>50%) and low methoxyl (<50%) (Ghaffari *et al.*, 2007; Jug *et al*., 2012). Some authors have reported the formation of complexes between chitosan and pectin, two polyelectrolytes of opposite charge. The formation of these complexes has shown different behaviors (viscosity, zeta potential) in contrast when using only one polymer (Tsai *et al*., 2014). Also, it has been reported the use of this complex as a carrier for some drugs like vancomycin, aceclofenac, and carvedilol (Bigucci *et al*., 2008; Hagesaether *et al*., 2009; Kaur and Kaur, 2012)(Debandi *et al*., 2016).

b) Plasticizers

A plasticizer is a substance, which increases the flexibility and processability of polymer materials by lowering the glass transition temperature (Tg). As a minor component of polymeric drug delivery system has not been strictly defined. Even liquid drugs can serve as plasticizers

as well as structural water in the hydrophilic polymer (Snejdrova and Dittrich, 2012). Compatibility of the plasticizer with the polymer and plasticization efficiency are important factors when selecting the ideal plasticizer. According to solubility with water, plasticizers can be classified as hydrophilic and hydrophobic. Hydrophilic plasticizers are characterized by being substances with good biocompatibility, can be easily eliminated from the organism and some are components of metabolic processes. Appropriate plasticizers for dosage forms formulations has been declared in the 35th edition of the United States Pharmacopoeia (USP 35,2011) (Snejdrova and Dittrich, 2012)

In particular, glycerol has been incorporated in most of the hydrocolloid films (Vieira *et al.*, 2011). High boiling point (290°C) and high hydrogen bonding ability of glycerol (**Fig. 6**), leads to a good interaction with chitosan macromolecules (Debandi *et al*., 2016). Also, has been reported that glycerol resulted more suitable for chitosan films than ethylene glycol and propylene glycol (Vieira et al., 2011).

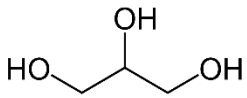


Fig. 6 Structure of Glycerol

Active ingredient

Buccal films for local application, not only play a role as a protective barrier, but also as an antiseptic, anti-inflammatory, antibacterial, antifungal, analgesic, among others, depending on the drug/bioactive used. Generally, from 5% to 30% w/w of the active ingredient can be incorporated in the buccal film (Radha *et al*., 2013; Raghavendra Rao *et al*., 2013).

In order to incorporate a lipophilic bioactive into the polymeric matrix, micronization, milling, emulsification (O/W) can be performed, this will improve dissolution and solubility, by reducing the size and increasing the surface area of particles of drug/bioactive (Radha *et al*., 2013).

I. Manufacturing methods of buccal films

There are different manufacturing processes to develop polymeric films, among which the solvent casting technique and the hot melt extrusion technique are the most used for buccal films.

a) Solvent casting technique

In this technique, the polymer is primarily dissolved in the solvent system. The polymeric solution is set aside for some time to remove any entrapped air and poured in a flat surface (such as a Petri plate), so the film can be formed by solvent evaporation **Fig. 7** (Chinna et al., 2011).

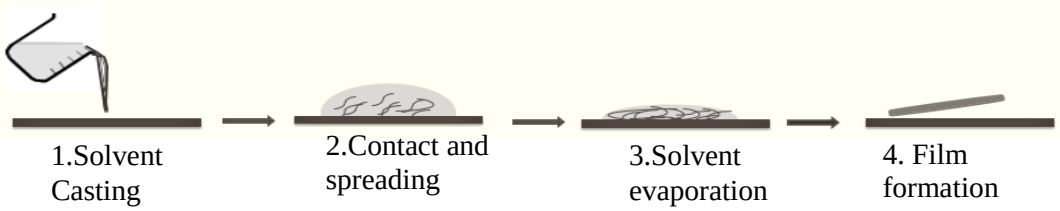


Fig. 7 Steps of film formation by solvent casting technique

b) Hot melt extrusion technique

In the hot melt extrusion technique; no solvents and water are necessary. A blend of the ingredients of the films is molten and forced through an orifice to yield a more homogeneous material in different shapes such as granules, tablets or films **Fig. 8**.

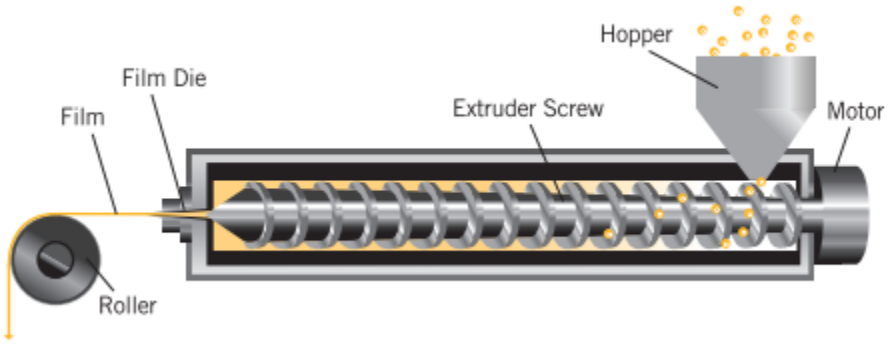


Fig. 8 Film formation by hot melt extrusion technique. Retrieved from (Particle Sciences, 2010)

I.3.4 Emulsions

Emulsions are widely used as drug delivery system, in parental, oral, and topical routes. This system consists of at least two immiscible liquids, with one of the liquids being dispersed as small spherical droplets in the other (McClements et al., 2007). Two major categories of emulsions are oil-in-water (O/W) and water-in-oil (W/O). In the O/W emulsions, oil droplets are dispersed in the aqueous phase, while W/O emulsions consist of water droplets, dispersed in an oily medium (McClements, 2010).

Also, according to their particle size, emulsions can be categorized into three groups: macroemulsions (0.1-100µm), nanoemulsions (20-100nm) and microemulsions (5-50 nm). Macroemulsions and nanoemulsion are thermodynamically unstable. Physically, nanoemulsions and microemulsions are transparent unlike macroemulsions which are turbid and opaque (McClements, 2010).

Emulsions can be characterized by their particle size distribution, the polydispersity index, and zeta potential, together they can determine the physicochemical properties of the final product, like optical characteristics, rheology, stability and flavor (Bouyer *et al*., 2012; McClements, 2010).

The particle size distribution (PSD) represents the fraction of particles within different size groups. PDS it is often reported as a measure of the central tendency (mean) and a measure of the width of the distribution, which is indicated through the polydispersity index (PdI) (McClements, 2010). Samples with PdI values below 0.1 or slightly higher are considered monomodal distributions and can be used for comparative purposes. Distributions with PdI values above 0.5, cannot be used the single mean of particle size to characterize the emulsion(Malvern instruments, 2004a).

Around each particle, there is an electrical double layer composed of an inner region called the stern layer (counter-ions are strongly attached to the particle) and an outer region called diffuse layer (co-ions are presents). The electric potential tends to cero out of the diffuse layer, since the concentration of positive and negatives ions reached the equilibrium. The point where the Stern layer and the diffuse layer meet is known as the zeta potential, its magnitude can be a very useful representation of the electrical characteristics of an emulsion and can serve as an indicator of stability. Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable (Malvern instruments, 2004b; McClements, 2010).

Some of the reasons to encapsulate lipophilic bioactive are that offer protection against environmental stress and degradation, mask unpleasant smells and enhance its penetration (Bouyer *et al*., 2012). Naturally, emulsions tend to break down over time due to physicochemical phenomena like gravitational separation, flocculation, coalescence, and Ostwald ripening (McClements *et al*., 2007). To avoid this mechanism, surface-active molecules are used, these amphiphilic molecules consist of a hydrophilic and a lipophilic part, that adsorbs at the interface of the two phases during homogenization. Depending on the emulsifier selected, the mechanism of o/w emulsions are most of the time by stearic stabilization, nevertheless, others are stabilized by electrostatic and stearic repulsion (Ushikubo and Cunha, 2014). Stearic stabilization arises from physical barriers while electrostatic stabilization is based on the mutually repulsive forces present in the ionic emulsifiers.

When using biopolymer for emulsion stabilization, the droplet sizes of particles are in the order of 1µm or more and formation of nanoemulsions with them is more difficult (Bouyer *et al*., 2012), such is the case of Arabic gum (GA). This biopolymer contains both protein and polysaccharide subunits. Three main fractions are present in this hetero-polysaccharide: the arabinogalactan (AG), the glycoprotein (GP) and the arabinogalactan-protein (AGP) fractions. The last one is responsible for the emulsifying properties of GA. In emulsion systems, the polysaccharide blocks of the GA takes a "water-blossom structure" conferring the macromolecule an amphiphilic character (proteinaceous fractions of the gum would embed in the oil phase, while the carbohydrate component would extend out from the surface into the aqueous phase) and thus favoring the adsorption at the oil-water interface. Steric hindrance and electrostatic stabilization are present in the GA emulsions (Bouyer *et al*., 2012).

On the other hand, small monomeric surfactants adsorb at the interface more quickly, which contributes to the production of small droplets (Bouyer *et al*., 2012). Polysorbate 80 (T80) and hydroxylated lecithin (LecH) are good examples of these. T80 is one of the most non-ionic, synthetic surfactant used, because of its high hydrophilic and lipophilic balance value (HLB-15) suitable for o/w emulsions. Also, its low molecular weight makes it more efficient in minimizing droplets size (Ghosh *et al*., 2013a).

Soybean lecithin is a plant-derived surfactant and has been used in the food, cosmetic and paints industry. It contains complex mixtures of compounds, such as phospholipids, which is the surface active agent in lecithin. The structure of phospholipids has saturated and unsaturated

fatty acids, this has an impact on the degree of hydrophilicity and consequently in the adsorption behavior at the oil-water interface. The introduction of hydroxyl groups into the unsaturated fatty acid chain has been reported to improve the adsorption behavior at the oil-water interface (Nyankson *et al*., 2016).

Nowadays, technologies of high and low energy are used to produce emulsions. In the low energy methods, emulsions are obtained as a result of phase inversion or through spontaneous emulsification, some drawbacks of this method are that high quantities of surfactants are required and sometimes the process can be slow. Colloidal mills, ultrasonicator, high pressure and high velocity homogenizers are some of the high energy equipment's used for the emulsification process (Ghosh *et al*., 2013b; Hashtjin and Abbasi, 2015; Sugumar and Singh, 2016).

The ultrasonication is a high energy equipment that makes possible the reduction of particle size, through sound waves generated by a sonotrode that triggered mechanical vibrations, favoring the collision between particles, and thus decreasing particle size and polydispersity index (Ghosh *et al*., 2013a, 2013b). The concentration and surfactant to oil ratio (ROS), besides ultrasonication time and power input, are some of the parameters that can be modulated in order to obtain emulsions with different physicochemical characteristics. Ultrasonication has begun to be applied for the development of EOs based emulsions because the equipment is easy to use, is economical and can produce stable emulsions with a very small particle size (Salvia *et al.*, 2014). **Table 2** shows different delivery systems with essential oils, mainly nanoemulsions produced by ultrasonication technique.

Table 2. Application of essential oils in delivery systems

Essential oil	Formulation of delivery system	Application	Author
***Ocimum basilicum* (Estragole)**	Nanoemulsion by ultrasonication (750W), Tween 80	Antibacterial activity *E.coli*	(Ghosh *et al*., 2013a)
Cymbopogon citratus	Nanoemulsion by ultrasonication (400W), Sodium alginate + Tween 80	Antibacterial activity *E.coli*	(Salvia *et al*., 2014)
Citrus Sinensis	Nanoemulsion by ultrasonication (750W), Tween 80	Anti-yeast activity *S.cerevisiae*	(Sugumar and Singh, 2016)
Cinnamomum zeylanicum	Nanoemulsion by ultrasonication (750W), Tween 80	Antibacterial activity *B. Cereus*	(Ghosh *et al*., 2013b)
Origanum vulgare	Nanoemulsion by ultrasonication (750W), Tween 80	Antibacterial activity *Listeria monocytogenes Salmonella Typhimurium E.coli*	(Bhargava *et al*., 2015)
Lippia graveolens	Pectin coating for tomato fruits	Antifungal activity *A. alternata*	(Rodriguez *et al*., 2016)
Lippia graveolens	Pectin films by casting technique	Antibacterial activity *E. coli, S.aureus, L. monocytogenes*	(Alvarez *et al*., 2014)
	Essential oils in plastic film	Preserve goods, horticulture products	US20040034149 A1
Cinnamon and clove	Cassava starch film by casting technique, glycerol, clay nanoparticles. Sucrose ester as an emulsifier.	Antifungal activity *P.commune, E.amstelodami*	(Souza *et al*., 2013)
Cinnamon	Fish gelatin films by casting technique, glycerol. Tween 80 as an emulsifier.	Antimicrobial activity *E.coli, S.aureus, A.niger, R.oryzae, P.variotii*	(Wu *et al*., 2017)
Rosemary	Chitosan films with Tween 80 and essential oil by casting technique	Antimicrobial activity *Listeria monocytogenes P.putida, S. agalactiae, E.coli, Lactococcus lactis*	(Abdollahi *et al*., 2012)

II. Definition of the project

Chitosan and pectin can form films on their own from solutions with suitable solvents. In addition, polymer complexes can be generated between the two because of their opposite charges, which may result in the improvement of some physical properties of interest in mucosal films, such as the degree of water uptake and mucoadhesion. The characteristics of these biopolymers provide indications of their possible application to the buccal mucous as a protective barrier and as a carrier of antimicrobial agents

III. Justification

There are more than 400 different types of oral cavity disorders which are usually treated by local therapy (Sao *et al*., 2009). Within these, buccal candidiasis is an infection in the oral cavity caused by the opportunistic pathogen *C. albicans.* This fungus is normally found in the oral cavity, nevertheless in the suppressed or compromised patients can rapidly invade the oral tissues and spread to the lungs or esophagus (Sao *et al*., 2009). One of the main problems with current drugs (triazole derivatives such as fluconazole and ketoconazole) used to combat *C.albicans* is the develop resistance (Manzano *et al*., 2008; Rodrigues *et al*., 2010; Whaley *et al*., 2017). Besides, there are reports from the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) indicating that ketoconazole tablets can cause severe liver damage, adrenal gland problems and harmful interactions with other drugs (FDA, 2013; EMA 2013).

For the aforementioned, new alternatives are required to address this problem. In the last year, there has been an increase in the number of publications related to the use of EOs, due to their multiple properties (antioxidant, antiseptic, anti-inflammatory, antibacterial, antifungal, analgesic) (Donsì and Ferrari, 2016)

LGO or Mexican oregano essential oil is considered one of the most potent antimicrobial agents against a broad spectrum of microorganisms, and as a consequence to its multicomponent composition, the resistance of *C. albicans* turns out to be more difficult since inhibition of this pathogen is possible to different mechanisms.

Unfortunately, the volatility of the phenolic compounds in LGO and its hydrophobicity makes it difficult to incorporate into other materials that serve as delivery vehicles.

In the present work, it is proposed to develop an LGO based emulsion embedded in a particular dosage form with to natural polymers (Chitosan/Pectin), in order to obtain a mucoadhesive buccal film able to inhibit *C. albicans* growth in a localized way.

IV. Hypothesis

1. Different ratio mixtures of chitosan and pectin polymers will allow obtaining a polymeric matrix with the potential to be applied in the buccal mucous in comparison of the films formulated by a single polymer.

2. Emulsifier properties of *Lippia graveolens* essential oil emulsion have an impact on the inhibitory effect of *C. albicans*.

V. Objectives

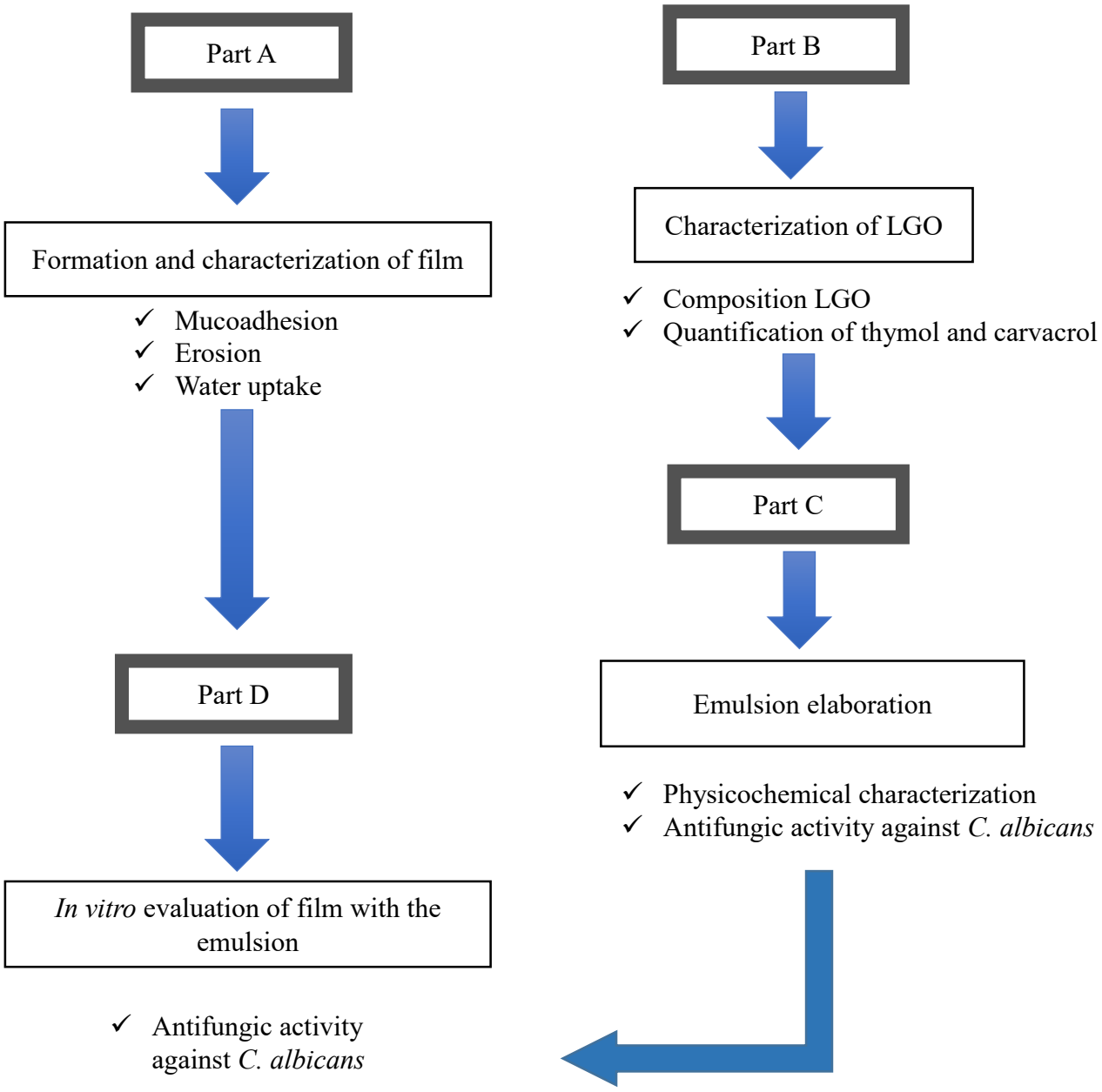
V.1 General objective

Develop of a polymeric (Chitosan/pectin) buccal film as a carrier of a *Lippia graveolens* essential oil emulsion and evaluate against *Candida albicans*

V.2 Specific Objectives

1. Develop and characterize the physical properties of films made with pectin and chitosan.
2. Identify and quantify the bioactive thymol and carvacrol in the *Lippia graveolens* essential oil by gas chromatography coupled with mass spectrometry (GC-MS)
3. Evaluate the effect of the emulsifying material of the *Lippia graveolens* emulsion in their physicochemical properties
4. Evaluate *in vitro* the antifungal activity against *Candida albicans* of the *Lippia graveolens* emulsion by itself and in the polymeric film.

VI. General Methodology



VII.1 Formation and characterization of the films

VII.1.2 Preparation of chitosan/pectin complex mucoadhesive buccal films

Films were prepared by solvent casting technique. Chitosan (CH) and pectin (P) were dissolved separately overnight in 1% acetic acid solution and distilled water, respectively. The final concentration of solutions was 1.5%. Solutions were made with an immersion mixer at different ratios CH:P (100:0, 75:25, 50:50, 25:75,0:100), pH of the mixture was adjusted to 5.5. Different concentration of plasticizer was added (glycerol 0%, 10% and 30%). Seven grams of the final mixtures were poured in Petri dishes (5 cm in diameter) and dried at 40 °C for 5 hours to evaporate the solvents. Formed films were detached carefully from Petri plates with tweezers and were conditioned to a water activity of 0.22, at 25 °C with potassium carbonate for further studies.

VII.I.3 *In vitro* water-uptake and erosion studies for the films

For water-uptake studies, the same methodology of Hagesaether *et al*., (2009) was followed with some modifications. Phosphate buffer (PBS) at pH 6.8 was used as simulated saliva. Complete films were weighted (W_1) and immersed in 40 mL of PBS for four hours in a water bath (37 °C, 20 rpm). Excess of water was removed, and the hydrated films were re-weighed (W_2). To determine if any erosion had taken place, hydrated films were dried at 100 °C all night and weighed (W_3). The procedure was made by quadruplicate. Water uptake (%) and erosion (%) were determined according to Eq.1 and Eq.2 respectively.

$$Water\ uptake\ (\%) = \frac{(W_2 - W_1)}{W_1} \cdot 100 \ldots\ldots\ldots\ldots\ldots\ldots Eq.1$$

$$Erosion\ (\%) = \frac{(W_3 - W_1)}{W_1} \cdot 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq.2$$

VII.I.4 Mucoadhesion test for the films

The methodology of Jug *et al*., (2012) with some modifications, was followed to determine mucoadhesion properties of the films. Porcine buccal mucous (PBM) was used as a model substrate; time for complete detachment of the film was recorded. PBM was obtained from a local slaughterhouse. The tissue was washed with distilled water and phosphate buffer. Portions

of the porcine buccal tissue were cut (area ~ 6 cm^2) and fixed with cyanoacrylate to a glass support. Prepared films were cut and placed on the PBM by applying light pressure with a fingerprint for 10 s. The supports with the PBM and the film were immersed into beakers with 40 ml of PBS maintained at 37 °C, under 20 rpm. The experiment was performed for five hours and five replicates.

VII.2 Chromatographic analysis of the *Lippia graveolens* essential oil

Chromatographic profile of LGO (Nextipac, Jalisco) was developed by GC-MS by using a gas chromatograph Hewlett Packard, 5890 SERIES II coupled to a Mass Selective Detector model 5972 SERIES (Palo Alto, Ca).

Compounds were separated in a polyethylene glycol HP-FFAP capillary column (50 m × 0.22mm × 0.33µm). Ionization was applied (EI 70 eV) in SCAN mode. Helium was used as carrier gas at a flow rate of 0.8 mL min^{-1}. The volume of sample injected was 0.2 µL with a split ratio of 1:115. Injector and detector temperature were 250 °C and 280 °C respectively. Oven program, was set from 80 °C to 185 °C, increasing 5 °C min^{-1}, held 9 min, then the temperature was increased at a rate of 10 °C min^{-1} until 220 °C and held for 5 min. Compounds in the sample were identified by comparing their mass spectra with that provided by the Wiley library 275L and by comparison of the relative retention times of some authentic compounds (Sigma-Aldrich >95%) injected under the same analytical conditions.

Quantification was based in percentage area, by taking the automatic electronic integration data produced by the Chemstation version B.01.00 software.

Identical chromatographic conditions were also applied for thymol and carvacrol quantification in the essential oil analyzed in SIM mode, from an external standardization. For thymol, the concentrations used for the calibration curve were of 2419, 4032, 6048, 8063, 10079, and 12095 ppm. For carvacrol were of 2390, 3984, 5975, 7968, 9959, and 11951 ppm. A diluted sample of LGO in ethanol (1:20) was used in order to be within the range of calibration curves. The measurements were performed in duplicate.

VII.3 Emulsions preparation and characterization

VII.3.1 Emulsions formation

Oil in water (O/W) emulsions were prepared with a mixture of LGO (Nextipac, Jalisco) and neobee in a ratio 1:1. The concentration of oil phase and the emulsifier-oil ratio (SOR) was 10 % and 0.5 respectively. Three emulsions with different emulsifier agent (Gum Arabic; GA (Nexira, Encapcia,), Polysorbate 80; T80 (Sigma-Aldrich, Tween 80), and hydroxylated lecithin; LecH (Cargill) were prepared. First, the coarse emulsions were obtained after mixing oil and aqueous phase at 9,500 rpm (ULTRA-TURRAX T 25 Germany) for five min at room temperature. After that, each coarse emulsion was homogenized using an ultrasound homogenizer (Fisher Scientific^{TM}, FB50, U.S.A.) with a 13 mm stainless steel probe with 50% of amplitude for 15 min. The homogenization temperature was controlled with a circulation water bath (Julabo, FP50, Germany) at 40 ± 1 °C.

VII.3.2 Quantification of thymol and carvacrol in the emulsions

The quantification of thymol and carvacrol in the emulsions was carried out under identical chromatographic conditions as for the quantification based on external standardization for the pure essential oil, described in section **VII.2.**

VII.3.3 Droplet Size Distribution, Polydispersity Index (PdI) and Zeta potential

The droplet size distribution and the polydispersity index (*PdI*) were obtained by dynamic light scattering technique in a Zetasizer Nano-ZS90 (Malvern Instrument, U.K). All emulsions were diluted 1:100 with distilled water and poured into the disposable cuvettes type ZEN0118. All measurements were carried out in duplicate and at 25 °C. Results were reported as the mean with the standard deviation. Tukey test was applied to find differences among means using a p-value = 0.05. The zeta potential was determined using electrophoretic light scattering equipment Zetasizer Nano ZS90. The measurements were carried out using the universal dip cell (ZEN1002, Malvern Instrument, UK) at 20 °C. The zeta potential is correlated the emulsion droplets velocity into an electric field using the Smoluchowsky model. The zeta potential was calculated from the average of two measurements of the diluted emulsion, and the results were reported as the mean and standard deviation

VII.3.4 Stability of emulsions

Stability studies were realized by storing the three emulsions with their controls at 5 °C, 25 °C and 40 °C. It was then observed for any phase separation or creaming. Also, change in droplet diameter was studied at different intervals of time. Tukey test was applied to find differences among means using a p-value = 0.05.

VII.3.5 Antifungal activity of emulsions against *Candida albicans*

An overnight culture of *C. albicans* (ATCC® 10231™) was used to inoculate new medium, then yeast was grown (37 °C and 250 rpm) until reached an optic density of 0.3 at 660 nm in YPD broth (peptone, glucose, yeast extract from Sigma-Aldrich). At this point, different volumes (0.9, 1.9, 3.75, 7.5, 15, 30, 60 µL) of emulsion were added to the test culture (2 mL) and incubated at 250 rpm and 37 °C for 16 h. An aliquot (50 µL) of the test culture was withdrawn after incubation and inoculated into agar YPD. Appropriate dilutions were made and viable colonies were counted after incubation at 37 °C for 24 h. Two controls were used, one without any treatment and the other with 60 µL of the emulsion without the essential oregano oil. Ketoconazole was used for comparative purposes (0.3 µg, 0.15 µg, and 0.075 µg).

VII.4 Antifungal activity of films+emulsion against *Candida albicans*

Disk diffusion assay was followed according to Alvarez et al., (2014) with some modifications. An overnight culture of *C. albicans* was used to inoculate new medium. Then yeast was grown (37 °C and 250 rpm) until reached an optic density of 0.3 at 660 nm. An aliquot (50 µL) of the test culture was inoculated and spread in YPD and immediately after that, selected films from mucoadhesion test were impregnated with emulsions and placed on the agar. A film with a blank emulsion (without bioactive) was used as a control.

VIII. Results

VIII.1 Films characterization

VIII.1.2 *In vitro* water-uptake and erosion of the films

Water uptake and erosion were evaluated for all the films prepared at different polymer ratios and concentrations of glycerol (**Fig. 9, Fig. 10**). Films consisting of merely pectin polymer disintegrated instantly in the PBS, this was also observed by Kaur and Kaur, (2012). Pectin as a hydrophilic polymer lets the liquid penetrates into the film, and after hydration and swelling, the gel-sol material goes into solution and erodes. Similarly, the chitosan films after some time in contact with water changed to a gel-like form when no glycerol was added, the film broke apart without reaching the swelling equilibrium, this was in accordance with Debandi *et al*., (2016). Chitosan films with 30% of glycerol maintained its integrity after four hours of hydration **(Fig. 11)**.

As the glycerol concentration increased, a reduction in water uptake for CH: $P_{75:25}$ films and $CH:P_{100:0}$ films was observed, most likely due to a clustering effect, where hydrogen bonds between the glycerol molecules involving the ones already linked to chitosan, were generated, giving place to a less porous film (Cui *et al*., 2011; Debandi *et al*., 2016).

There was no significant difference (p-value>0.05) between CH:$P_{25:75}$ and CH:$P_{50:50}$ films, these ones got the least water uptake values, likely because of the high degree of interaction between chitosan and pectin. The similar swelling behavior for CH:$P_{50:50}$ films at pH=5 were reported by Bigucci *et al*., (2008).

Besides glycerol concentration, polymers ratio also impacts in water uptake, which increased at a higher amount of chitosan, this matches results of Hagesaether *et al*., (2009) who developed films, using LM or HM pectin with chitosan. The increase in water uptake when more chitosan is added can be explained by the repulsion of protonated free amino groups that let the water molecules enter into the film structure.

In order to see if there was a loss of mass from the film, the erosion test was carried out. It was found that all films reached around 40% of erosion, even though integrity form of mixed films remained, after being immersed in PBS for 4 hours (Fig. **9**). No significant differences (p-value >0.05) were found in erosion values when varying glycerol concentration, excepting CH:$P_{50:50}$ film, where erosion tend to increase as increasing glycerol concentration (**Fig. 10)**.

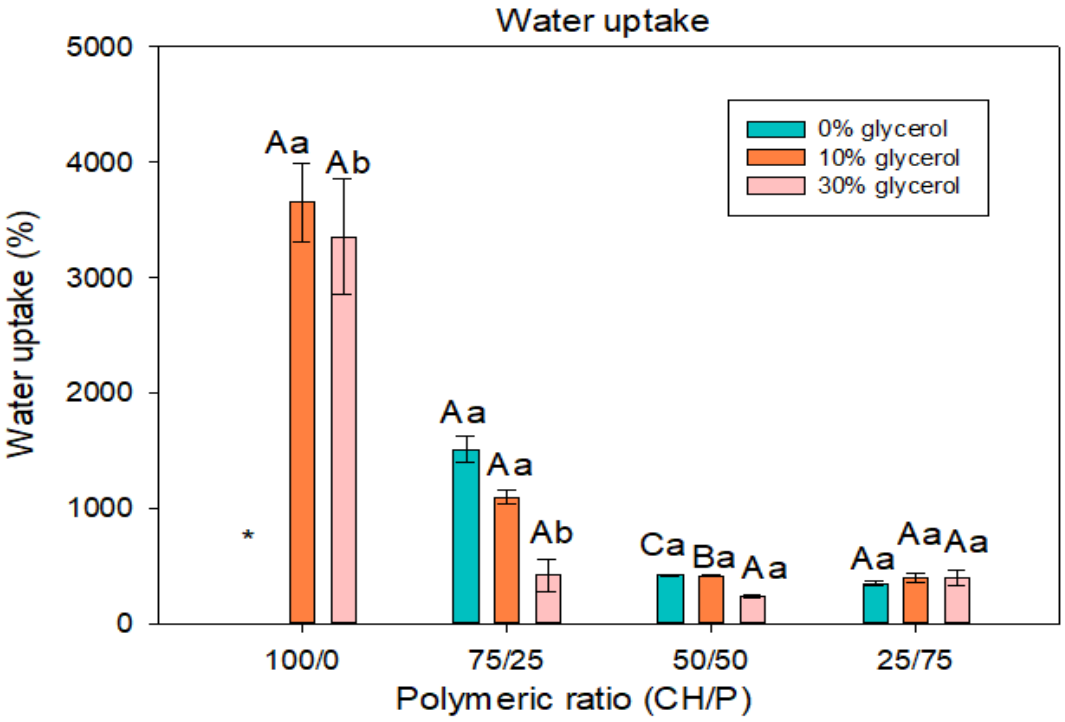


Fig. 9 Water-uptake of the films. Common capital letters are not significantly different (p>0.05) between glycerol levels. Common lower case letters are not significantly different (p>0.05) between polymer ratio. (mean ± SD, n=4)

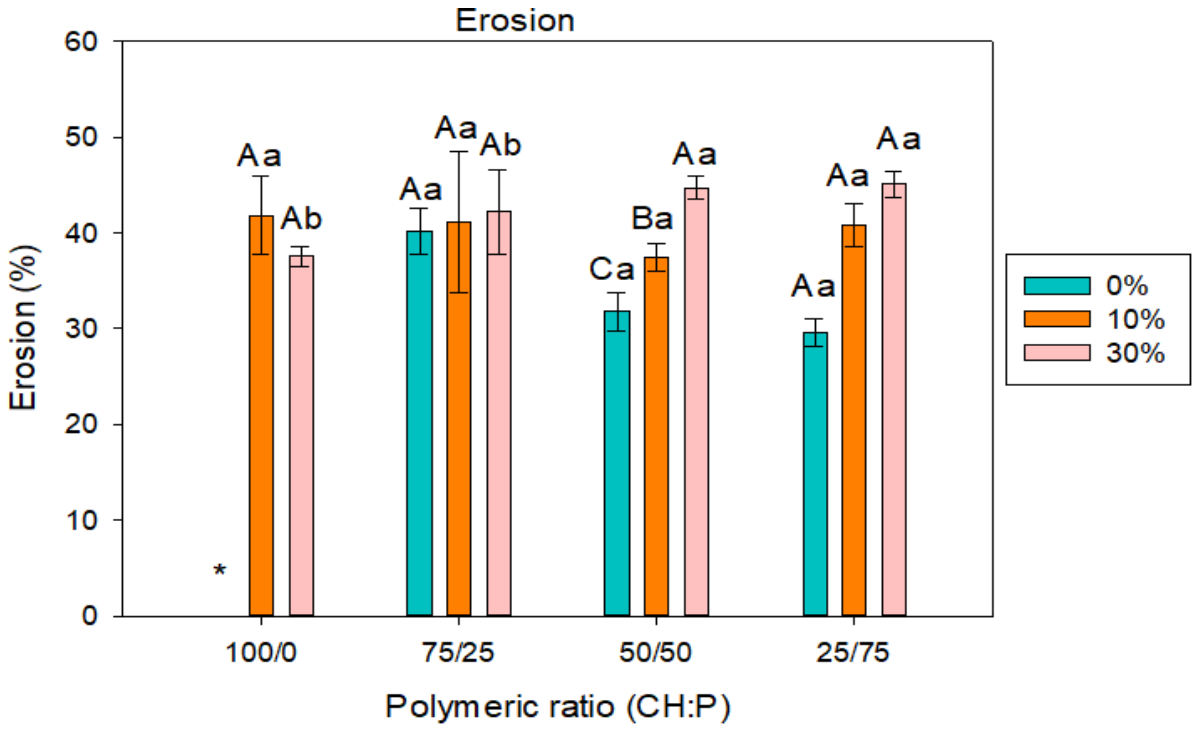


Fig. 10 Erosion of the films. Common capital letters are not significantly different (p>0.05) between glycerol levels. Common lower case letters are not significantly different (p>0.05) between polymer ratio. (mean ± SD, n=4)

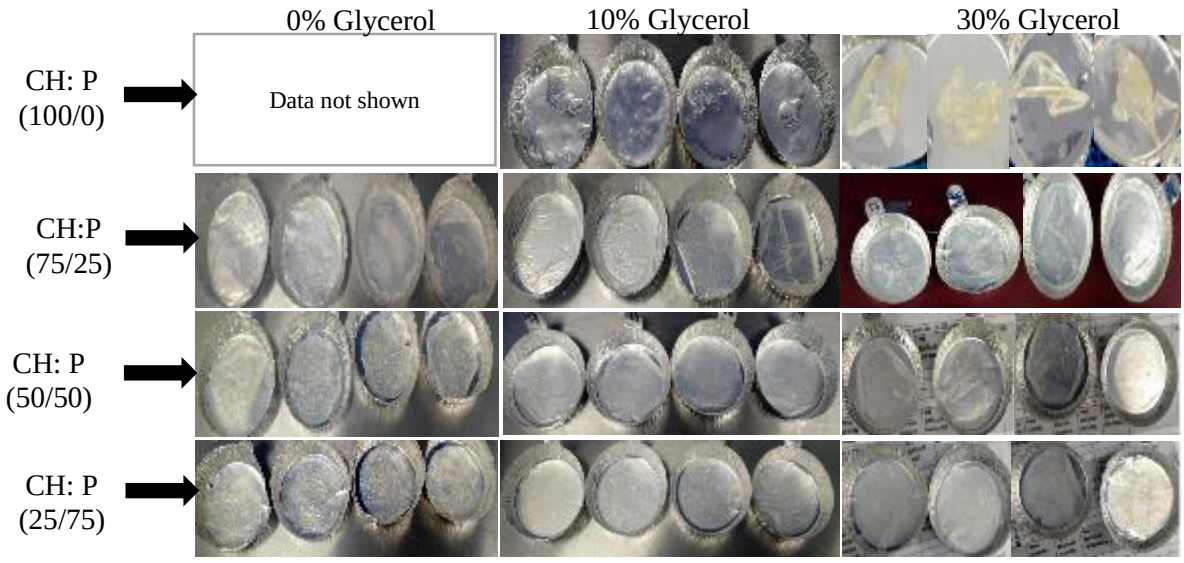


Fig. 11 Films after four hours in PBS (37 °C, 20 rpm)

VIII.1.3 Mucoadhesion of the films

Mucoadhesion is affected by the synergistic action of the properties of the polymeric delivery system (like water uptake, erosion, charge) and the biological environment (pH, enzymes) (Bigucci *et al*., 2008). In order to determine the films with best mucoadhesive properties, it was evaluated the number of films retained in the mucous during the five hours experiment, simulating the human oral cavity. Those experiments in which 80% of the films or more were retained to the mucous during at least 3 hours were considered successful. At the experimental conditions used (PBS pH 6.8), chitosan films without glycerol did not show mucoadhesive properties. The polyelectrolyte complex between chitosan and pectin is merely by electrostatic interactions, then pH is an important factor when analyzing the water uptake and mucoadhesion.

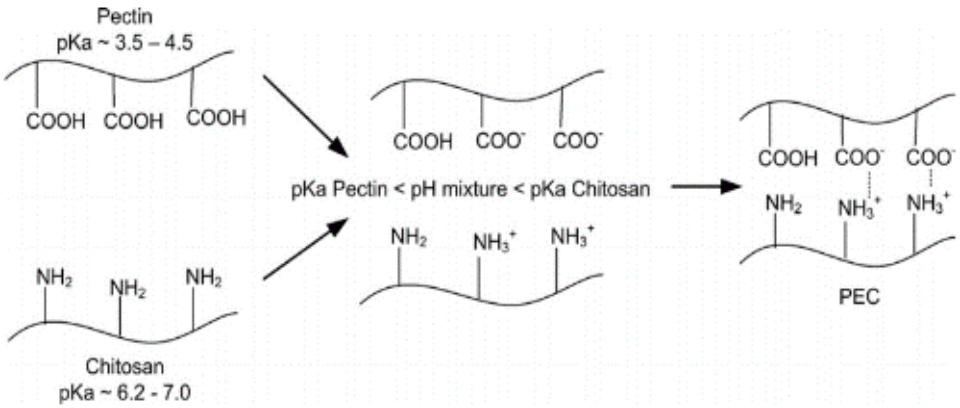


Fig. 12 Interaction between chitosan and pectin

In the pH range between the pKa´s of the two polymers, that is around 5-6, the polyelectrolyte complexes formation can occur more extensively since half of their ionic groups are ionized. In acidic medium, pectin is neutralized and free positive charge ($NH3^{+}$) appears in the system; while in a basic medium, chitosan is neutralized and free negative charge (COO^{-}) appears inside the matrix (**Fig. 12**) (Pandey *et al*., 2013).

Fig. 13 shows the mucoadhesion properties of the films without glycerol. Mucoadhesion of films consisting of 100% chitosan, could not be evaluated because of complete disintegration of the film in the PBS. All the replicates of CH:P $_{50:50}$ films were able to remain attached to the mucous surface for five hours, showing the best mucoadhesion properties of films without glycerol. The CH:P $_{75:25}$ film showed an excellent mucoadhesion properties, 80% of the films remained in the mucous for three hours **Fig. 13**.

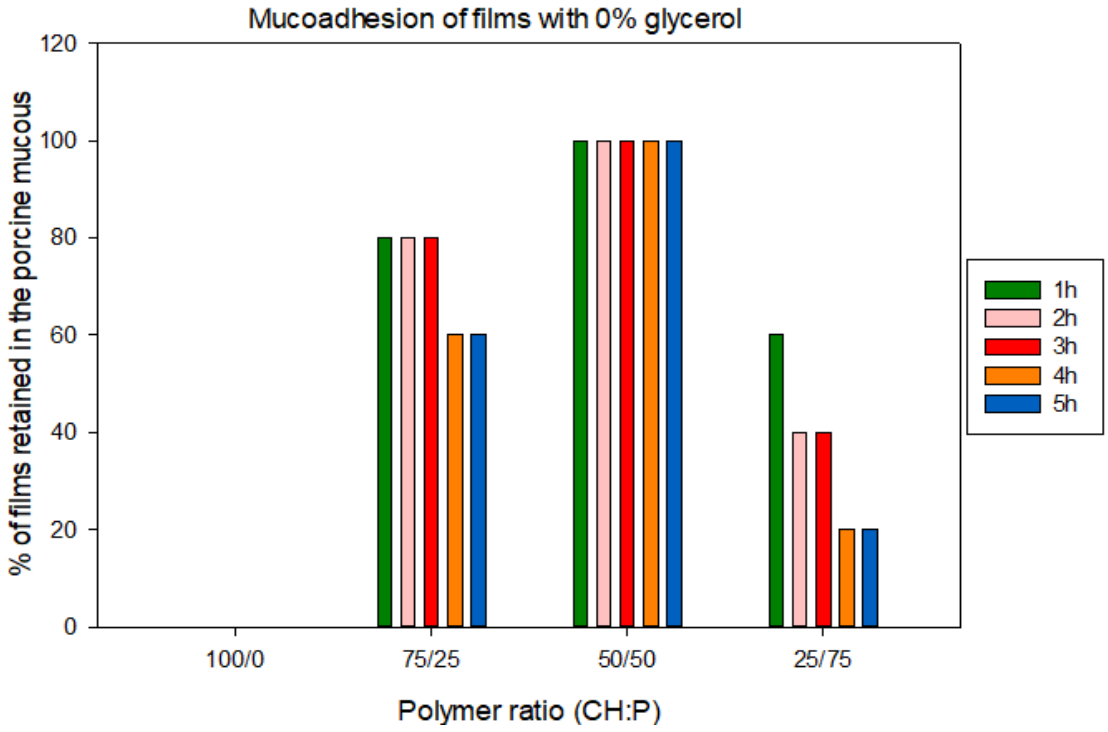


Fig. 13 Mucoadhesion of the film with 0% glycerol. N=5

When the concentration was increased to 10% of glycerol, the mucoadhesion of CH:P $_{25:75}$ films got reduced in contrast to CH:P $_{25:75}$ films with 0 % of glycerol. However, CH:P $_{75:25}$ and CH:P$_{50:50}$ films displayed a good mucoadhesion property. The 80% of the films in both polymer ratios, remained in the mucous for five hours (**Fig. 14**).

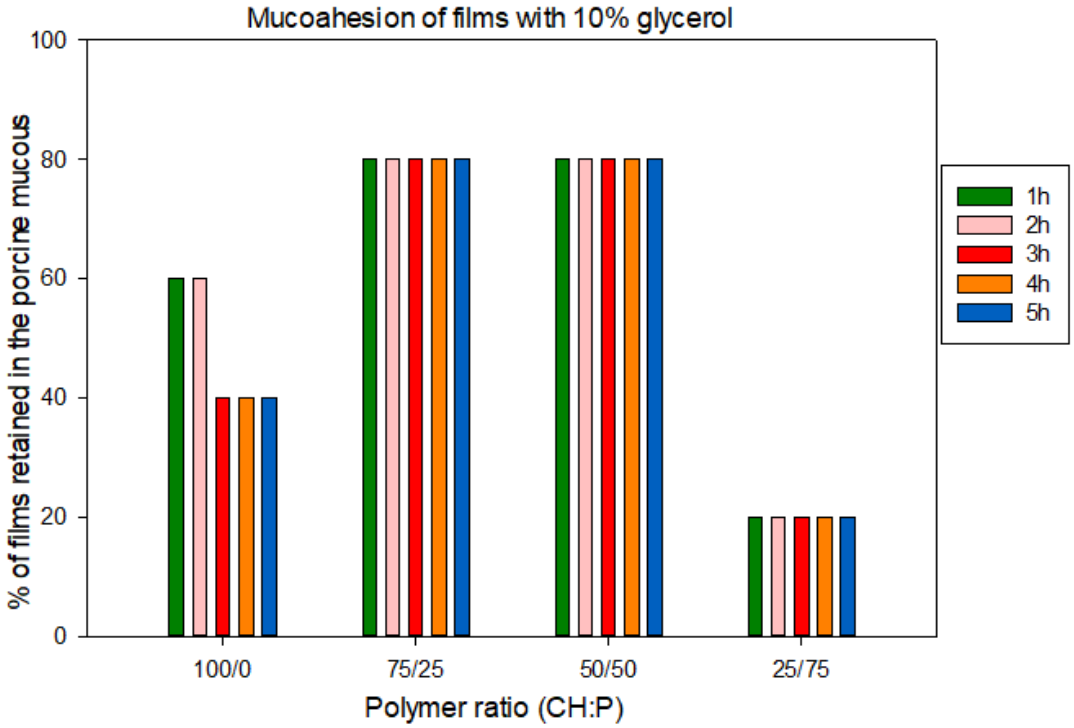


Fig. 14 Mucoadhesion of the film with 10% glycerol. N=5

Mucoadhesion properties of films were reduced with 30% of glycerol (**Fig. 15**), except CH $_{100:0}$ films. According to Abruzzo *et al*., (2012), films with more amount of chitosan, presented better mucoadhesive properties at pH 6.8, due to the interaction between positively charged amino groups of chitosan and negatively charged sialic acid (pKa 2.6) and sulfate residues of mucin glycoprotein. The results obtained in the present work were according to this, the CH:P $_{100:0}$ films with 30% glycerol and the CH:P $_{75:25}$ films with 0% and 10% of glycerol, proved to have good mucoadhesion. Nevertheless, a good mucoadhesion property observed for the films of CH:P$_{50:50}$ with 0% and 10% glycerol could be a result of some free protonated amino groups of chitosan, some available hydrogen bonding sites or the swelling ability. Bigucci et al., (2008) reported that swelling ability increases the mobility of molecules, facilitating the interpenetration and interaction with the mucous layer.

CH:P $_{75:25}$ films with 10% glycerol, CH:P$_{50:50,}$ films with 10% glycerol and CH$_{100:0}$ films with 30% glycerol were selected to be used for *in vitro* tests against *C. albicans*, because of their mucoadhesion properties. (VII.4 Antifungal activity of films+emulsion against Candida albicans).

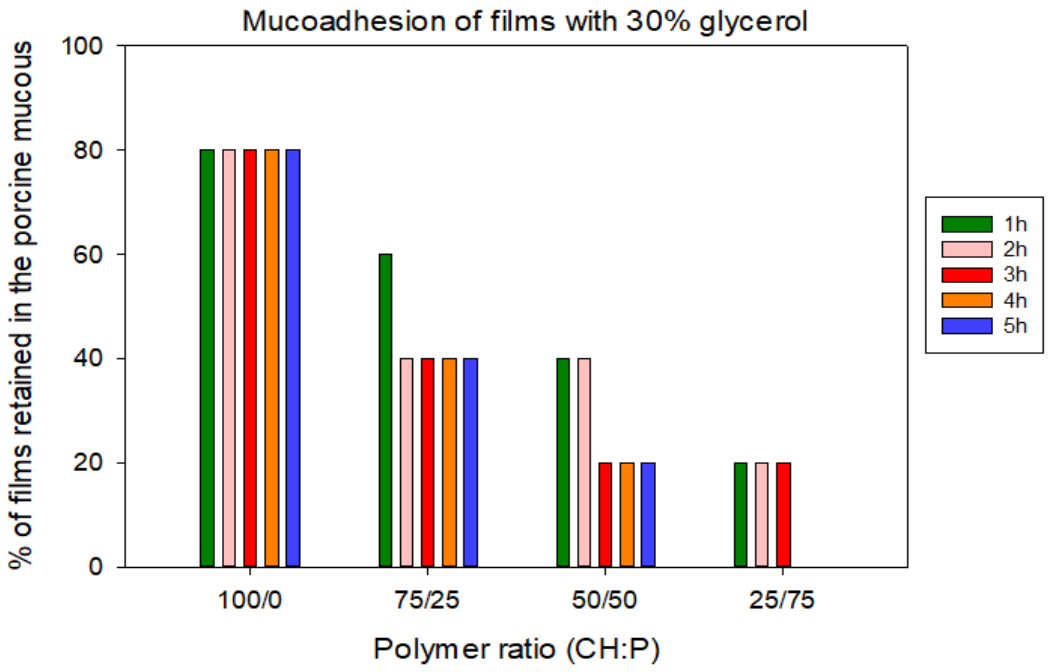


Fig. 15 Mucoadhesion of the film with 30% glycerol. N=5

VIII.2. GC-MS analysis of pure *Lippia graveolens* essential oil

Table 3 shows the volatile compounds identified with their respective retention times and abundance according to the percentage of the area determined by GC-MS in *LGO*. On the other hand, the Total Ion Chromatogram (TIC) of LGO analysis is shown in **Fig. 16**. A total of 30 volatile compounds were identified, representing 95% of the composition detected in the essential oil. Monoterpenes hydrocarbons, oxygenated monoterpenes, sesquiterpenes hydrocarbons, and phenols were some of the compounds groups found in the LGO. The essential oil was thymol chemotype. Three main components were identified: thymol (31.66%), carvacrol (14.57%) and p-cymene (18.72%). These results were similar to those reported by (Castillo *et al*., 2007) which also contained thymol (27.26%), carvacrol (12.3%) and p-cymene (14.16%) as the major components. Rodriguez *et.al* (2016) also quantified thymol (3.02%), carvacrol (47.41%) and p-cymene (26.44%) in LGO. Variations in the concentration of this components were expected, due to many factors that affect the composition of EOs, as the harvesting season, geographical sources and extraction method (Arana *et al.*, 2010; Castillo *et al*., 2007). Although thymol and carvacrol are considered the most potent antifungal and antioxidant compounds of oregano oil (Hernández *et al*., 2014; Rodriguez *et al*., 2016), p-

cymene may play an important role as an antagonist or as an enhancer on the activity of the essential oil (Botelho *et al.*, 2007). The concentration of thymol and carvacrol in SIM mode was 286.42 g/L and 118.92 g/L respectively (Appendix 1).

Table 3. Volatile compounds of *Lippia graveolens* essential *oil*

No[a]	RT[b]	Compounds	% Area[c]		
		Monoterpene hydrocarbons	LGO	Castillo *et.al*, 2007	Pino *et.al*,1989
1	4.30	α-Thujene	2.41	0.55	0.4
2	4.69	Camphene	0.63	1.01	1.7
3	5.09	β-Pinene	0.16	0.16	0.8
4	5.48	β-Myrcene	1.68	*	5.5
5	5.87	α-Terpinene	1.68	0.71	1.7
6	6.11	Limonene	1.35	1.45	3.5
7	6.82	γ -Terpinene	2.42	2.28	2.7
8	**7.29**	**p-Cymene**	**18.72**	**14.16**	40.6
		Oxygenated Monoterpenes			
9	6.35	1,8- Cineole	3.44	15.49	10.7
10	10.91	trans-Sabinene hydrate	0.22	0.26	*
11	12.49	Linalool	0.7	2.32	2.4
12	12.80	cis-Sabinene hydrate	0.21	2.32	*
13	13.99	Terpinen-4-ol	1.55	3.83	1.9
14	14.54	p-Menth-2-en-1-ol	0.14	0.08	*
15	16.11	Borneol I	1.25	2.98	0.5
16	22.25	Caryophyllene oxide	0.42	1.72	1.9
		Sesquiterpene hydrocarbons			
17	13.28	trans- α-Bergamotene	0.49	0.10	*
18	13.83	β-Caryophyllene	5.62	2.27	17.0
19	15.40	α-Humulene	2.97	3.07	10.5
20	16.43	β-Bisalobene	0.41	0.34	1.0
		Phenols			
21	13.59	Thymol methyl ether	1.28	*	*
22	19.26	Thymol acetate	0.44	0.12	*
23	19.73	Carvacrol acetate	0.15	*	*
24	**27.06**	**Thymol**	**31.66**	17.26	57.9
25	**28.08**	**Carvacrol**	**14.57**	12.30	20.6
		Carbonylic compounds			
26	10.36	1-Octen-3-ol	0.53	0.07	*
		Others			
27	10.52	p-Cimenil	0.14	0.02	*
28	18.24	Benzoic acid	0.18	0.05	*
29	18.95	Trans-carveol	0.11	0.15	*
30	21.07	Benzaldehyde	0.19	0.03	*

The three most abundant compounds are presented with bold letters. [a] Peak number of compound identified according (Fig. 16). [b] Retention time of compound. [c] Percentage area of compounds compared with other authors with Mexican LGO.

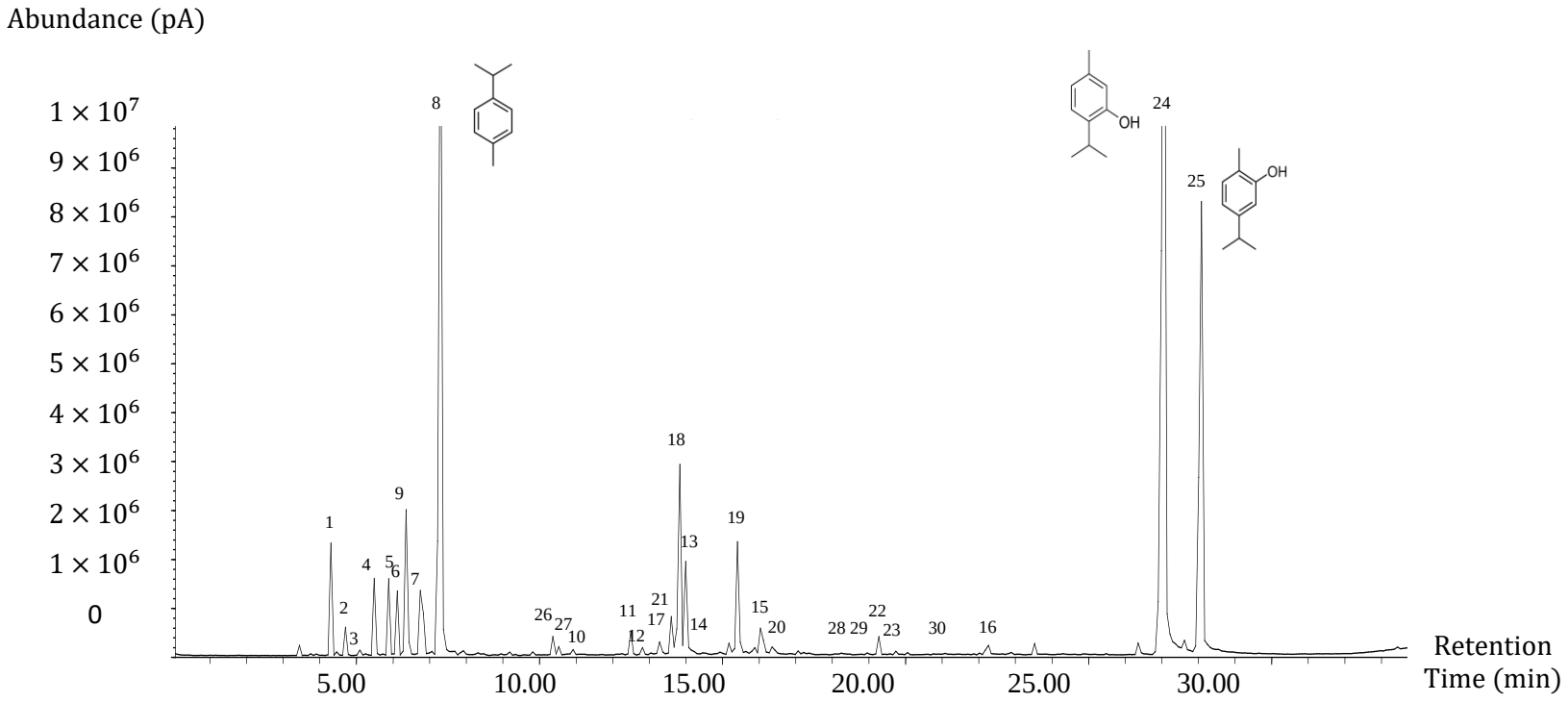


Fig. 16 –Total ion chromatogram (TIC)of *Lippia graveolens* essential *oil*

VIII.3 Emulsions characterization

VIII.3.1 Droplet size, PdI and Zeta potential

Droplet size of emulsions after ultrasonication are shown in **Table 4**. All emulsions had a monomodal behavior **(Fig. 17)**. Several authors have reported the use of T80 as surfactant because of its high-lipophilic balance (HLB-15) is favorable to form an oil-in-water emulsion with small particle size (< 200 nm) (Bhargava *et al*., 2015; Ghosh *et al*., 2013a; Hashtjin and Abbasi, 2015). As expected emulsion with T80 exhibited the smallest particle size (137.3±4.2 nm). These results correspond to those reported by Bhargava *et al*., (2015) where they obtained an average particle size of 148 nm and a polydispersity index of 0.3 using oregano oil (6 %). GA emulsions showed the highest particle size (1597±11.1 nm). Emulsions with hydroxylated lecithin show an average particle size of 179±5.5 nm. There are no reports of oregano essential oil emulsion using GA and hydroxylated lecithin by ultrasonication techniques, however, this study confirmed its ability to act as a natural polymer matrix to carry oregano essential oil. β-cyclodextrin, modified starch, and whey protein concentrate have been used as encapsulating materials for Mexican oregano essential oil. Other authors have obtained EOs nanoemulsions with smaller particle size, when using ultrasonicators with power input of 750 W. (Bhargava et al., 2015; Ghosh et al., 2013a, 2013b; Sugumar and Singh, 2016) pH (~5), density (~0.995) and refractive index (~1.35) were similar in all emulsions because the only surfactant was different between them.

Table 4. Emulsions Characterization

Emulsions	Z-average (nm)		PdI		Zeta potential (mV)	
	With LGO	Without LGO	With LGO	Without LGO	With LGO	Without LGO
T80	137.3 ± 4.2^{Bc}	193.4 ± 8.4^{Ab}	0.177 ± 0.024^{Aa}	0.215 ± 0.024^{Aa}	-27.3 ± 1.25^{Ac}	-26.1 ± 0.38^{Ac}
LecH	179 ± 5.5^{Bb}	208.5 ± 8.8^{Ab}	0.193 ± 0.009^{Aa}	0.161 ± 0.009^{Aa}	-70.3 ± 1.8^{Aa}	-64.7 ± 1.92^{Ba}
GA	1597 ± 11.1^{Aa}	1365 ± 21.4^{Ba}	0.156 ± 0.108^{Aa}	0.205 ± 0.153^{Aa}	-40.3 ± 0.53^{Ab}	-39.3 ± 0.98^{Ab}

Common capital letters are not significantly different (p>0.05) between columns. Common lower case letters are not significantly different (p>0.05) between rows. Data recovered from day 0. (mean ± SD, n=3)

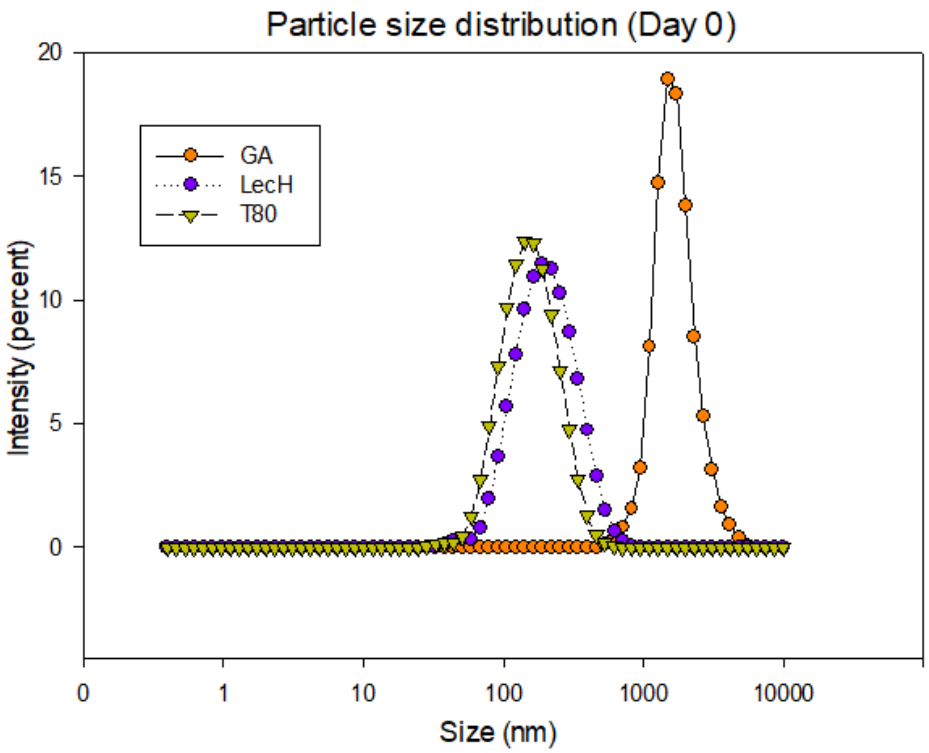


Fig. 17 Particle size distribution of T80, LecH and GA emulsions on Day 0

VIII.3.2 Stability of emulsions

T80 emulsion showed the smallest particles size at 5°C and 25°C of storage temperature, no significant differences (p-value > 0.05) were found in particle size from day 7 to day 28, indicating good stability of T80 emulsion at these temperatures. However, at 40°C there was a notable increase of particle size through (**Fig. 18)**. LecH emulsion was the most stable at all temperatures during the stability test (**Fig. 19)**. On the other hand, GA emulsions presented creaming since day 7 **(Fig. 20)**.

The smaller size and lower molecular weight of T80 and LecH surfactants allowed them to adsorb at the interface very quickly, contributing to the production of small droplets in comparison with GA, which is a biopolymer with a more complex structure, who tend to generate globules around 1µm (Bouyer *et al*., 2012).

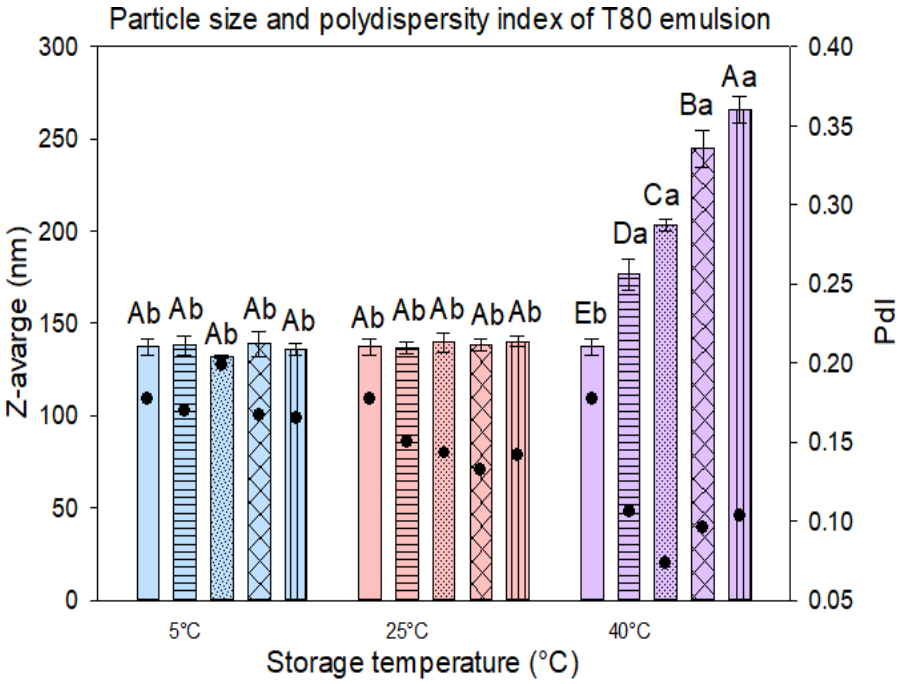


Fig. 18 Stability of T80 emulsion by particle size and polydispersity index. Common capital letters are not significantly different (p>0.05) between temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28

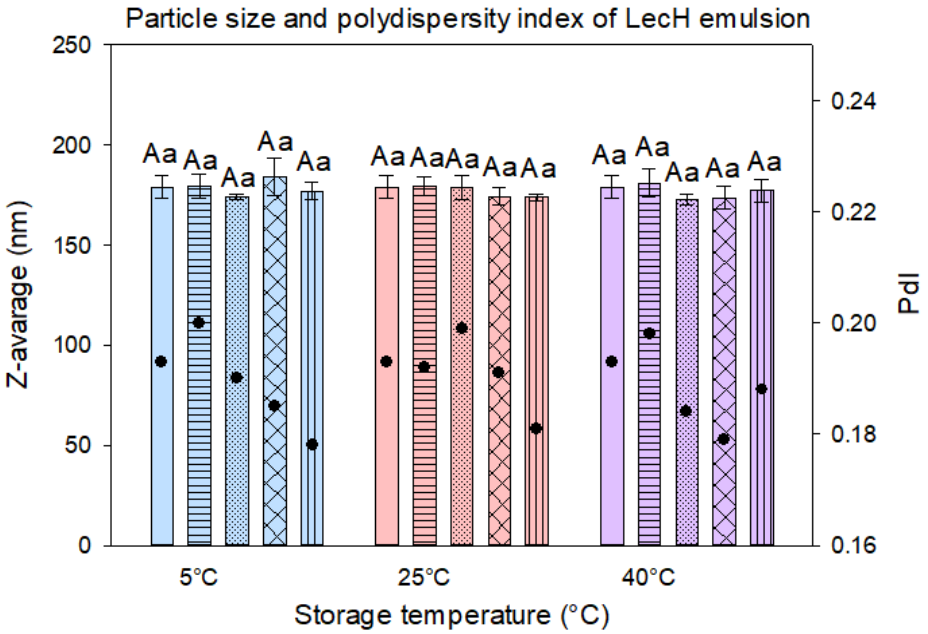


Fig. 19 Stability of LecH emulsion by particle size and polydispersity index. Common capital letters are not significantly different (p>0.05) between temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28

Different stability behaviors were expected from emulsions since each emulsifier used, has different physicochemical properties, and hence different stabilization mechanisms. Since T80 is a non-ionic surfactant, emulsion stability in this type of system is achieved by stearic stabilization (Ghosh *et al*., 2013a). On the contrary, stability in GA and LecH emulsions are a consequence of their negative charge.

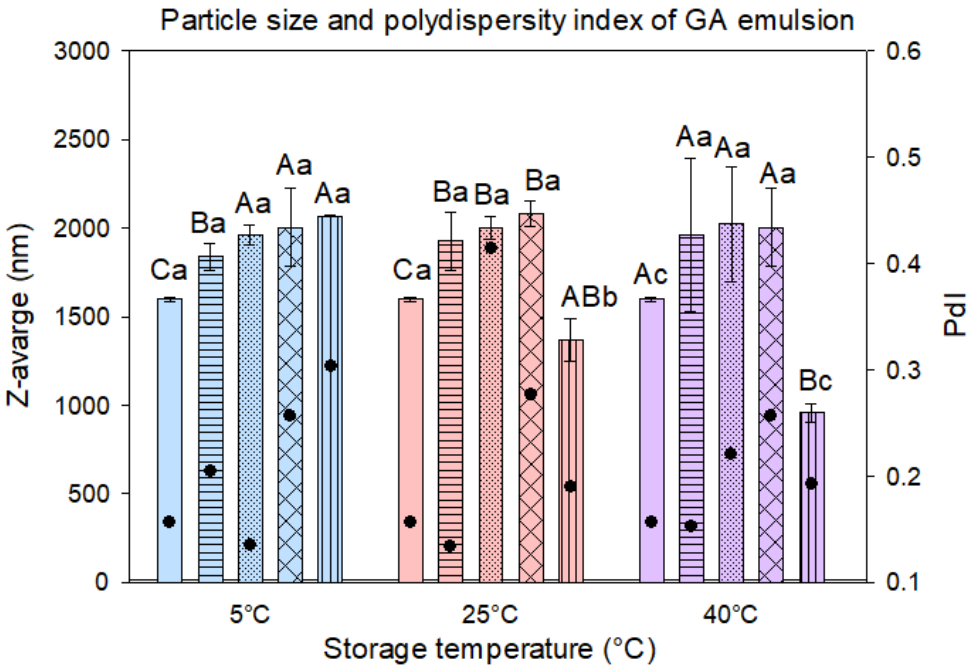


Fig. 20 Stability of GA emulsion by particle size and polydispersity index. Common capital letters are not significantly different (p>0.05) between temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28

The stability of emulsions by zeta potential was evaluated since it has been reported that the droplet aggregation is prevented mainly by electrostatic stabilization (Bouyer *et al*., 2012).). The zeta potential of the three emulsions ranged from -80 to -20 mV. Particularly, zeta potential values of GA emulsions during the stability test were maintained around -40 mV, some variations were observed mainly between days (**Fig. 21)**

Also, the zeta potential of LecH emulsions had minimal variations between days. Values of zeta potential were around -70 to -80 mV (**Fig. 22).**

Particles with zeta potential values above +30 mV and below -30 mV are usually considered stable systems because of strong repulsion forces (Ochoa *et al*., 2016). In accordance with this statement, LecH emulsion was the most stable. Although T80 is a nonionic surfactant, it also presented negative charge, this may be due to the free fatty acids and polar components in the oil phase (Bhargava *et al*., 2015) **(Fig. 23)**.

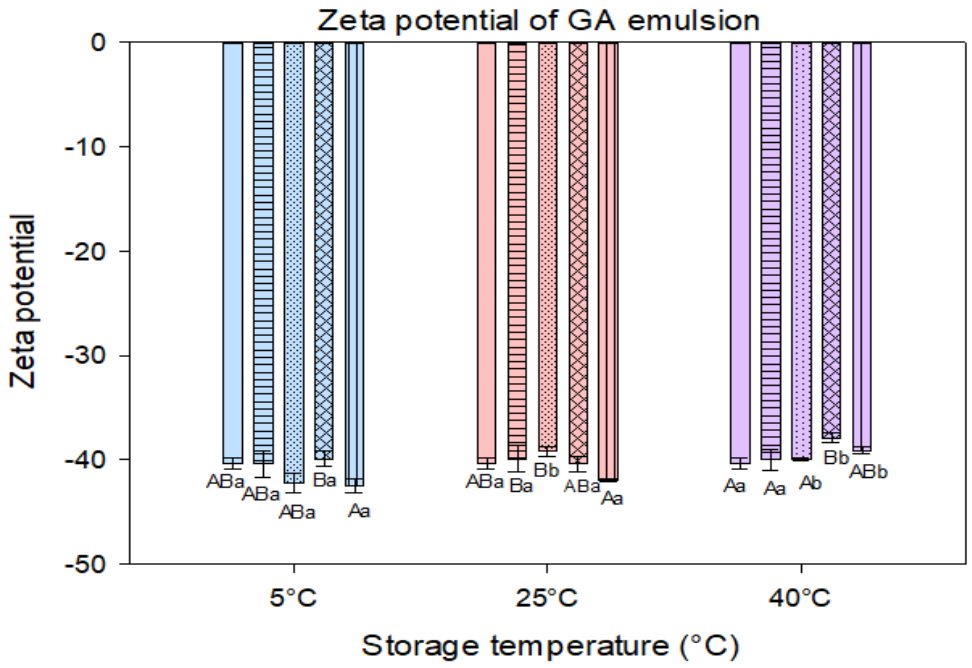


Fig. 21 Zeta potential of GA emulsions. Common capital letters are not significantly different (p>0.05) between temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28

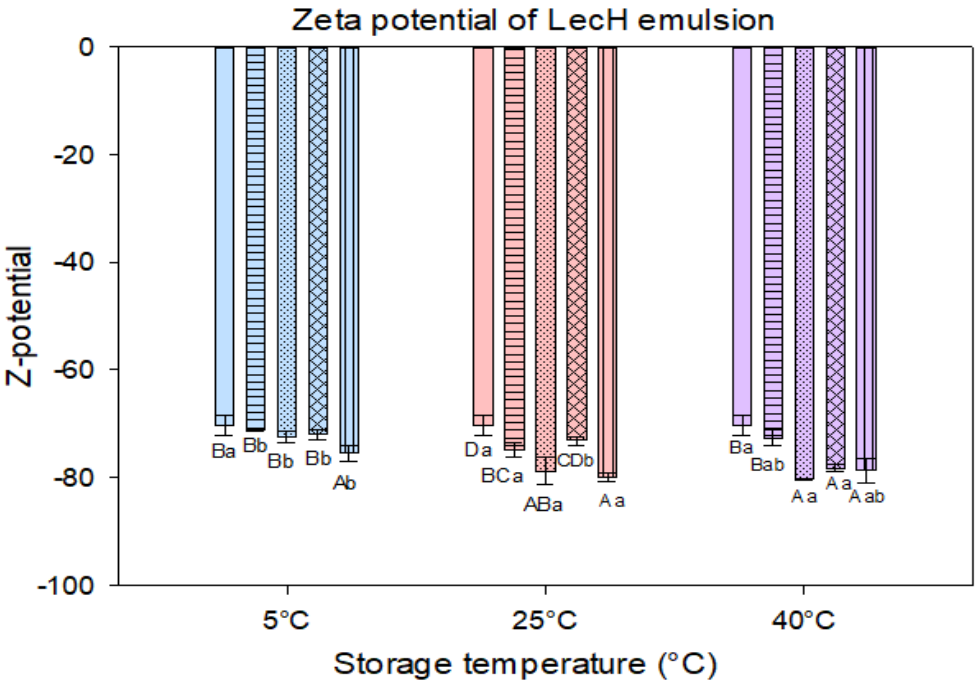


Fig. 22 Zeta potential of LecH emulsions. Common capital letters are not significantly different (p>0.05) between temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28 days

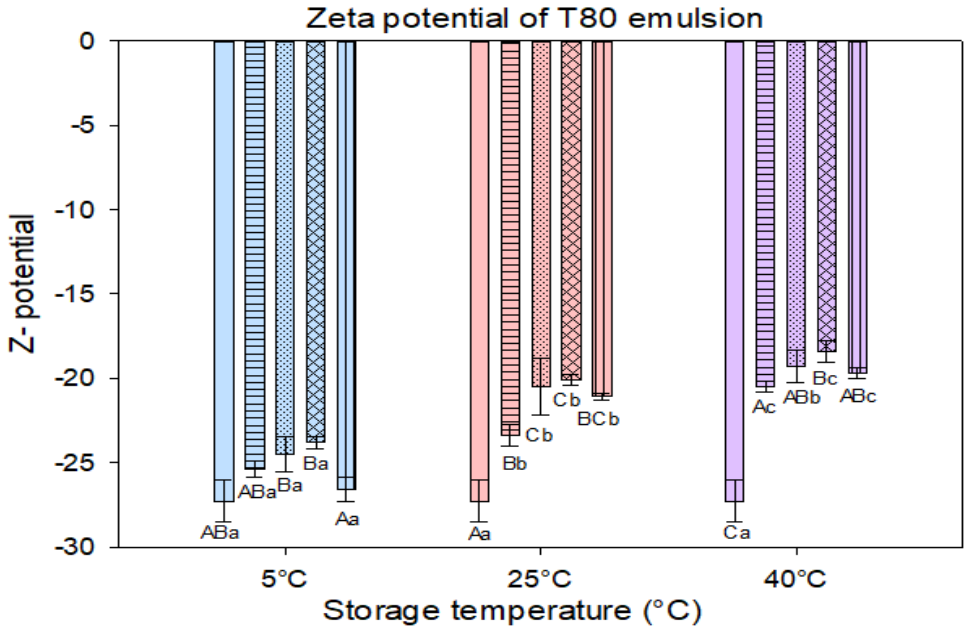


Fig. 23 Zeta potential of T80 emulsions. Common capital letters are not significantly different (p>0.05) n temperature groups. Common lower case letters are not significantly different (p>0.05) between days. Horizontal stripes day 7, black dots 14 days, rhomboid day 21, vertical lines day 28 days

VIII.3.2 Quantification of thymol and carvacrol in emulsions

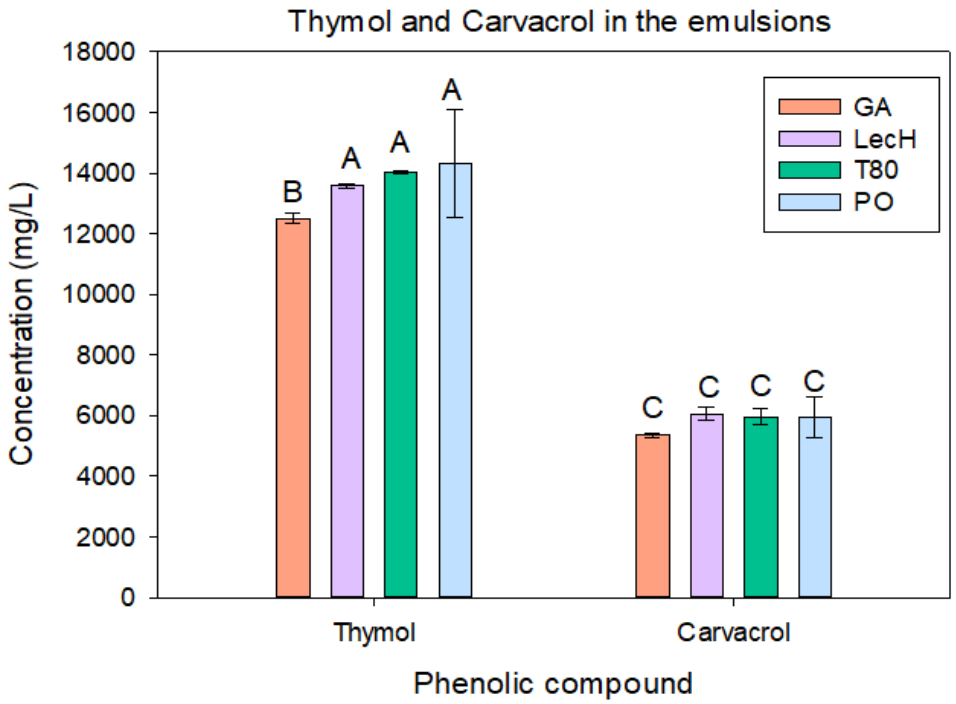


Fig. 24 Quantification of thymol and carvacrol from *Lippia graveolens* essential oil into the emulsions. Error bars represent standard error of the mean. Columns having common letters are not significantly different ($p > 0.05$)

Fig. 24 shows the concentration of thymol and carvacrol in each emulsion; there are no significant differences in carvacrol concentrations among all formulations (p-value >0.05). There was a significant difference in thymol concentration when comparing GA emulsion with pure oil (PO). Thymol concentration decreases around 10% in the GA emulsion compared with the PO. This can be explained by an incomplete separation of this compound due to the complex matrix or to the rupture of the emulsion due to the high temperatures used in the GC-MS

VIII.3.3 Antifungal activity against *C. albicans* of *Lippia graveolens* essential oil emulsions

Different degrees of inhibition were found between the three emulsions. At maximum concentration, GA and LecH emulsions proved to inhibit 100% of *C. albicans*. Below 3.5 µL of emulsions, there was no significant difference in the degree of inhibition of *C. albicans* (p-value

> 0.05). No significant differences were found between No tx and control, indicating no inhibitory effect by none of the polymers used as emulsifiers (GA, LecH, T80) at that concentration, thus inhibition of *C. albic*ans can be attributed only to the essential oil.

The antimitotic ketoconazole shows good anti-candida effect, in spite of using low concentration. Maximum inhibition was 97 % with 0.3 µg of ketoconazole.

Fig. 25 shows the log reduction of *C.albicans* with different volumes of LecH emulsion. With 7.5 µL of LecH emulsion (0.37 µL of oregano essential oil), there was around 1 log reduction in the viable cell compared to the controls. With 30 µL LecH emulsion there was an 8 log reduction in viable cells.

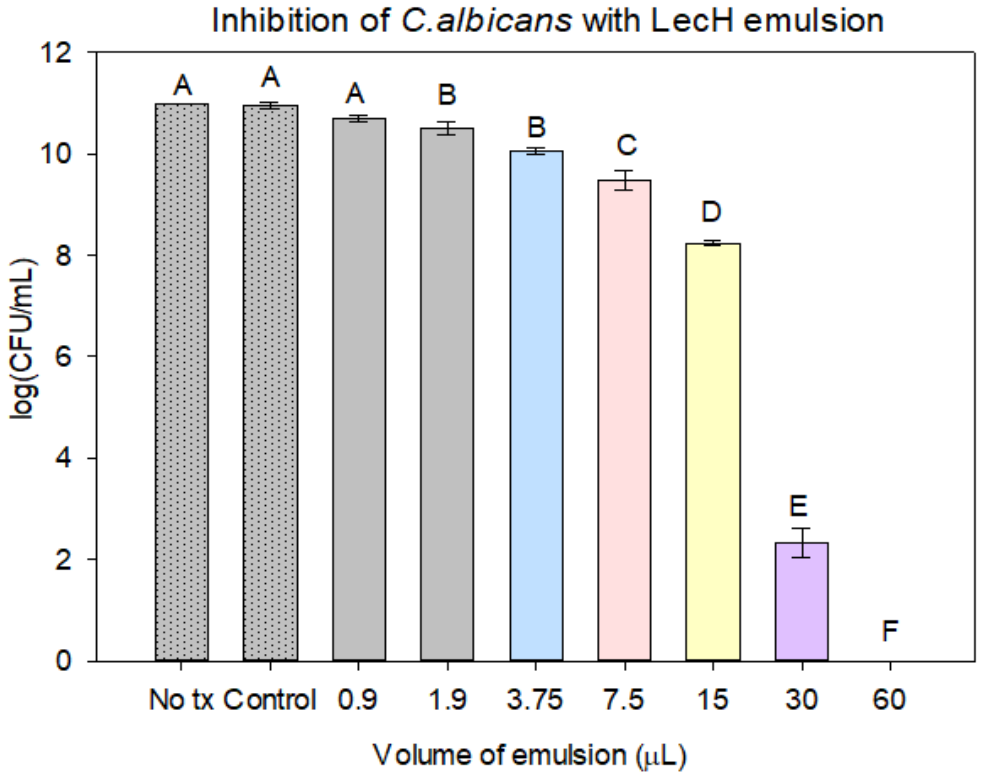


Fig. 25 Inhibition of *C. albicans* with the LecH emulsion. Error bars represent standard error of the mean. Columns having common letters are not significantly different (p >0.05)

It has been reported that antimicrobial properties of nanoemulsions are due to nano-size (<20-100 nm) of oil particles, because there is a high surface tension which can fuse and disrupt the membrane of microorganisms (Donsì and Ferrari, 2016; Ghosh *et al*., 2013a, 2013b). However, Buranasuksombat *et al*., (2011) obtained lemon myrtle oil particles from nano to micron size, without any synergistic effect observed between the oil and the particle size. Another

mechanism of nanoemulsions for antimicrobial inhibition was hypothesized by Ziani *et al*., (2011). Departing from the fact that the membrane of the yeast cells has negative charge, it would be expected that positively charged droplets would be attracted to their surfaces, whereas negatively charged droplets would be repelled. Conversely, they found an antagonist impact rather than a synergistic impact on the overall antimicrobial effect.

Guided by the literature, we expected T80 emulsion to be the most efficient; conversely, GA emulsion shown better effect, in spite of having the largest particle size and negative charge. 1 log reduction of *C.albicans* was reached with 7.5 µL of GA emulsion, 3 log reduction with 15 µL, and 5 log reduction with 30 µL (**Fig. 26**).

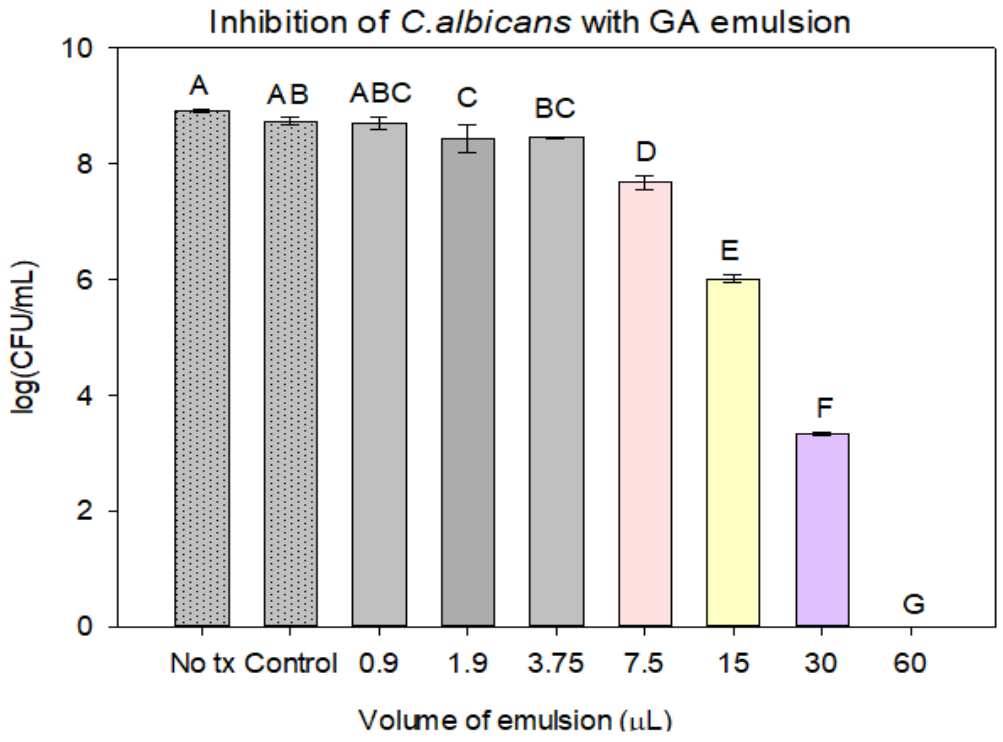


Fig. 26 Inhibition of *C. albicans* with the GA emulsion. Error bars represent standard error of the mean. Columns having common letters are not significantly different (p >0.05)

It is known that GA emulsion had a pH of 5, and the broth for *C. albicans* a pH around 6.5. It has been reported by Avadi *et al*., (2010) that Arabic gum polymer in media with pH values higher than 6.5 tend to swell and disturb the structure of nanoparticles, resulting in more porosity in the nanoparticle structure, what can be taken to a more bioactive release.

Likewise, in this work, it was not found a relationship between inhibition potential with smaller particles, therefore it is possible that inhibitory effect of each emulsion is related to the mechanism in which the emulsifier expose or interacts with the bioactive. For T80 emulsion (**Fig. 27**), 4 log reduction (99.99%) was obtained with the maximum volume evaluated and 1 log reduction with 30 µL of the emulsion.

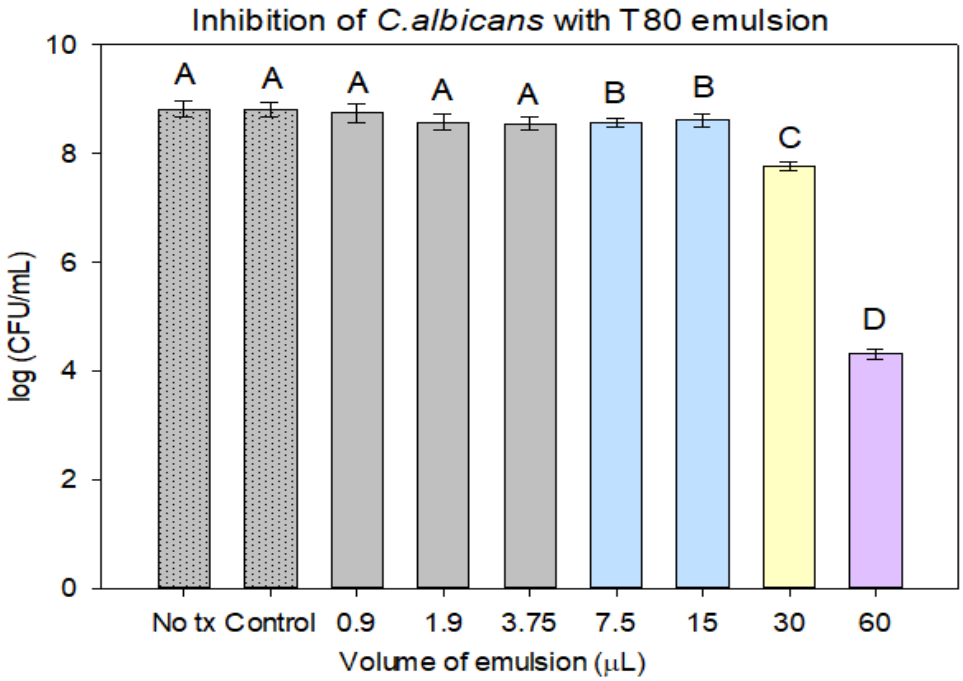


Fig. 27 Inhibition of *C. albicans* with the T80 emulsion. Error bars represent standard error of the mean. Columns having common letters are not significantly different (p >0.05)

The minimum lethal concentration (MLC) of thymol and carvacrol in the essential oil was determined in accordance with Ramos et al., (2012). The MLC was defined as the lowest concentration of bioactive at which microbial growth was prevented, and their initial viability was further reduced by at least 99.99% within 24h. As shown in **Table 5,** emulsion T80 was less effective in inhibiting *C.albicans* in comparison with the other two emulsions evaluated.

Table 5. MLC of thymol and carvacrol against *C. albicans*

	Thymol mg/ml	**Carvacrol mg/ml**
GA	0.187	0.08
LecH	0.203	0.09
T80	0.420	0.178

With this test, it can be concluded that the three emulsions had an inhibitory effect against *C. albicans*. However, further studies are required to elucidate the interaction between the bioactive and the emulsifier and find if there is a relationship with its biological effect.

VIII.4 Antifungal activity against *C. albicans* of films with *Lippia graveolens* essential oil emulsion

The disk diffusion assay let us verify if there was an inhibitory effect on the growth of *C. albicans* using the complete system (Polymeric film + emulsion), which were selected previously because of their mucoadhesion properties. Even when no diffusion halo was shown, it could be observed a better inhibition effect of the films with bioactive in comparison to the controls (**Fig. 28**).

One of the reasons for the lack of inhibition halos might be the incapability of the film material to diffuse the emulsion along the surface of the agar. Despite this result, we can conclude the inhibitory effect of the bioactive due to the visual comparison against the control.

It has been reported in the literature the inhibitory effect of chitosan against *C. albicans*, that explained the partial inhibition of the fungus with control films. It was not observed notable differences between control films when incrementing chitosan.

Some of the disadvantages of the results of *in vitro* studies is the difficulty to compare with other study, because of the different test methods, culture conditions, methods of extraction of essential oils, variations in the chemical compounds of essential oil due to different climatic conditions of the plant, among others (Yap *et al*., 2014).

In addition to that, the *in vitro* susceptibility of the microorganism to a particular bioactive does not guarantee the success of the clinical usage of the therapeutic agent, however, it is a good approach to obtain a natural formulation, with the possibility to be applied locally and to inhibit the opportunistic pathogen *C. albicans*.

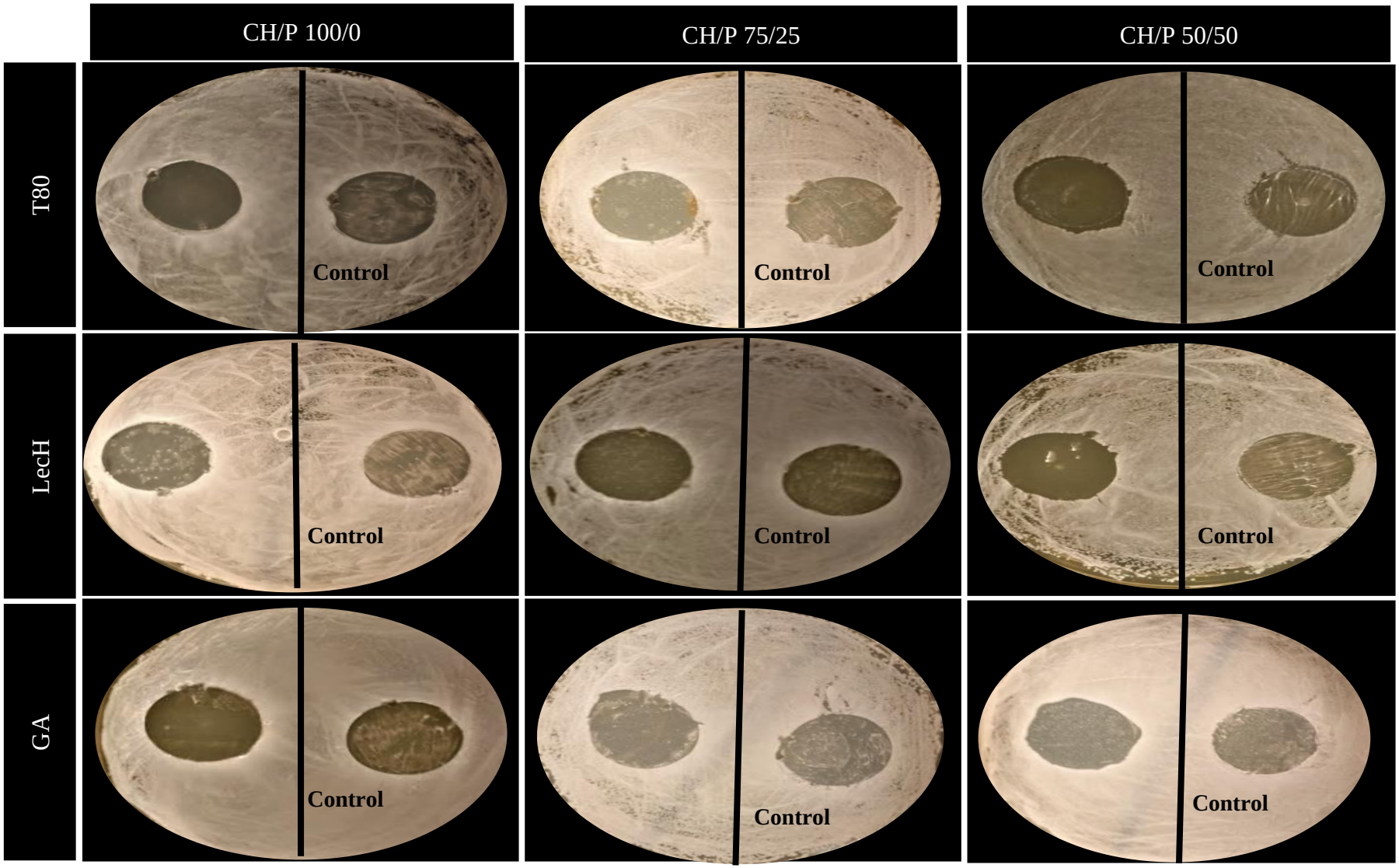


Fig. 28 *In vitro* inhibition of *C. albicans* with the films + emulsion

IX. Conclusions

- It was possible to identify and quantify the major compounds of a sample of *Lippia graveolens* essential oil from Nextipac, Jalisco.
- Using the ultrasonication technology it was possible to obtain *Lippia graveolens* essential oil emulsions. The use of three different emulsifiers (GA, LecH, T80) generates emulsions with different physicochemical properties. At the same time, the formation of a complex between chitosan and pectin in different relationships allowed the formation of films with different degrees of water uptake and mucoadhesion, confirming our hypothesis number one.
- The selected films in the present work were able to maintain its integrity form and the mucoadhesion property for five hours in the conditions simulating the human oral cavity.
- It was found that emulsions formulated with *Lippia graveolens* essential oil had *in vitro* antifungal properties against *C. albicans.* The three emulsions presented different degrees of inhibition, which confirmed the second hypothesis formulated in this work. The most efficient emulsion to inhibit *C. albicans* was with GA followed by LecH and finally with T80.
- It was possible to develop a system formed by a mucoadhesive film with an *Lippia graveolens* essential oil emulsion, which presented an inhibitory effect against *C. albicans* when compared with the same film without the bioactive

X. Perspectives

- It is recommended to complement the mucoadhesion test with other variables like force of detachment.
- Since GA emulsion was the most efficient inhibiting *C. albicans*, stability of this emulsion will be improved
- Monitoring the release of bioactive will allow knowing if more layers are required to control the delivery of bioactive
- For a local application, it would be convenient for the film dosage form, to add a backing layer, so the release of bioactive can be unidirectional.
- The emulsions should be evaluated against strains isolated from patients or resistant to the main drugs
- More studies are required to explain the different degrees of inhibition with the three emulsions.
- It is necessary to make a chromatographic analysis of *Lippia graveolens* essential oil after the emulsions formation.

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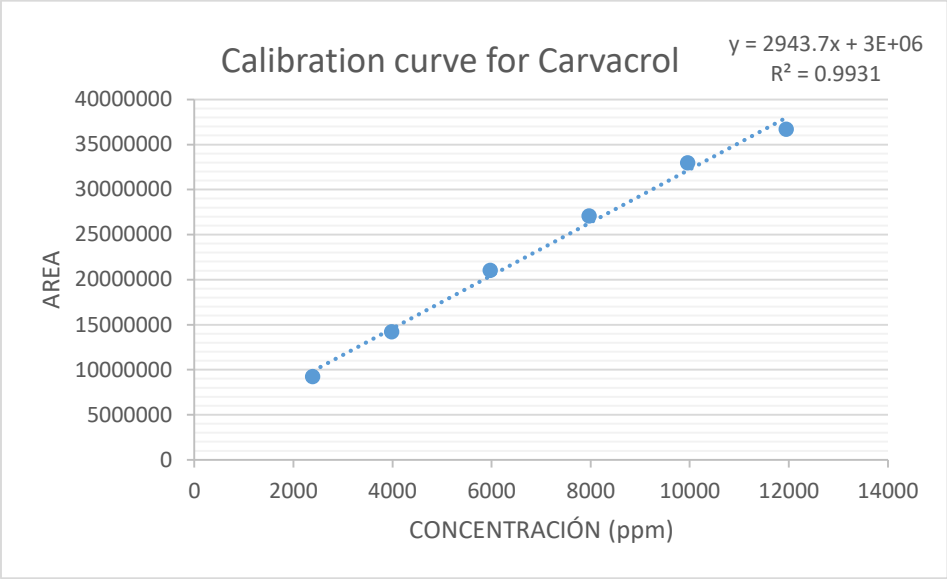
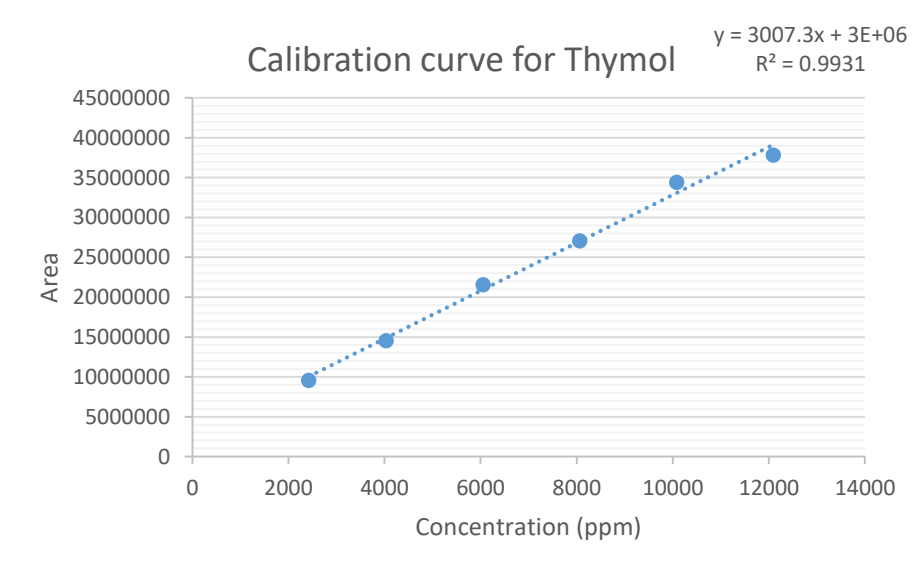
Appendices

Appendix 1. Calibration curves for quantification of thymol and carvacrol in the pure *Lippia graveolens* essential oil and in the emulsions





Appendix B. Particle size and Zeta potential of emulsions in the stability test

Table 1. Particle size GA

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	1840 ± 76^{Ba}	1962 ± 55.75^{Aa}	2028 ± 95.52^{Aa}	2066 ± 4^{Aa}
25	1597 ± 11.15^{A}	1732 ± 80.41^{Ba}	2003 ± 63.22^{Ba}	2083 ± 71.39^{Ba}	1368 ± 117.9^{ABb}
40	-	1964 ± 433.5^{Aa}	2023 ± 324.3^{Aa}	2005 ± 222.9^{Aa}	955.8 ± 51.71^{Bc}

Table 2. Particle Size LecH

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	179.6 ± 6.12^{Aa}	174 ± 1.44^{Aa}	184.2 ± 9.56^{Aa}	176.9 ± 4.36^{Aa}
25	208.5 ± 8.85^{A}	204.7 ± 4.05^{Aa}	178.8 ± 6.05^{Aa}	174.4 ± 4.06^{Aa}	173.8 ± 1.95^{Aa}
40	-	181.1 ± 7.36^{Aa}	173 ± 2.65^{Aa}	173.6 ± 5.650^{Aa}	177.3 ± 5.63^{Aa}

Table 3. Particle size T80

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	138.2 ± 5.11^{Ab}	132 ± 0.65^{Ab}	138.7 ± 6.86^{Ab}	135.7 ± 3.03^{Ab}
25	137.3 ± 4.22^{A}	136.7 ± 2.97^{Ab}	139.5 ± 4.89^{Ab}	138.4 ± 3.15^{Ab}	140.1 ± 2.76^{Ab}
40	-	176.6 ± 8.25^{Da}	203.3 ± 2.82^{Ca}	244.8 ± 9.86^{Ba}	265.9 ± 7.12^{Aa}

Tabla 4. Zeta potential GA

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	-40.4 ± 1.21^{ABa}	-42.2 ± 0.91^{ABa}	-39.9 ± 0.74^{Ba}	-42.5 ± 0.66^{Aa}
25	-40.3 ± 0.53^{AB}	-39.9 ± 1.25^{Ba}	-39.2 ± 0.47^{Bb}	-40.4 ± 0.78^{ABa}	-41.9 ± 0.12^{Aa}
40	-	-40 ± 0.97^{Aa}	-39.9 ± 0.15^{Ab}	-37.9 ± 0.46^{Bb}	-39.1 ± 0.35^{ABb}

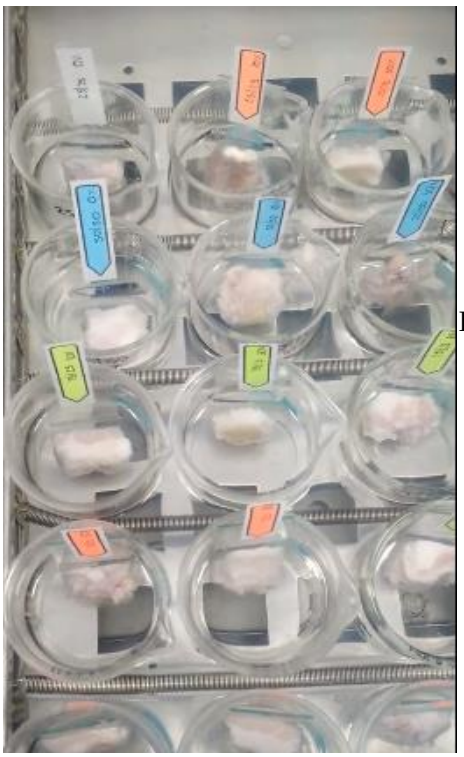
Tabla 5. Zeta potential LecH

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	-71.1 ± 0.17^{Bb}	-72.4 ± 1.12^{Bb}	-72 ± 0.86^{Bb}	-75.4 ± 1.45^{Ab}
25	-70.3 ± 1.8^{D}	-74.9 ± 1.133^{BCa}	-78.7 ± 2.66^{ABa}	-73.1 ± 0.8^{CDb}	-79.9 ± 0.89^{Aa}
40	-	-72.6 ± 1.46^{Bab}	-80.2 ± 0.11^{Aa}	-78.2 ± 0.64^{Aa}	-78.6 ± 2.25^{Aab}

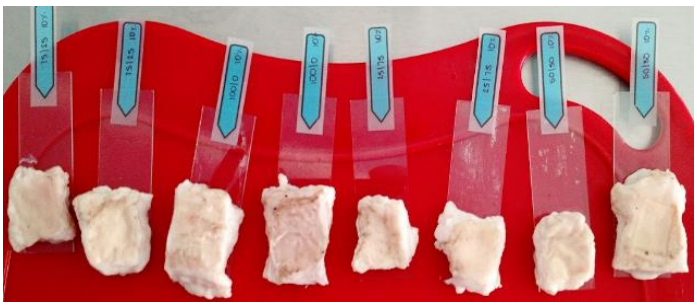
Tabla 6. Zeta potential T80

Temperature(°C)/storage time (day)	0	7	14	21	28
5	-	-25.4 ± 0.49^{ABa}	-24.5 ± 1.04^{Ba}	-23.8 ± 0.36^{Ba}	-26.6 ± 0.76^{Aa}
25	-27.3 ± 1.25^{A}	-23.4 ± 0.66^{Bb}	-20.5 ± 1.67^{Cb}	-20.1 ± 0.31^{Cb}	-21.1 ± 0.2^{BCb}
40	-	-20.5 ± 0.32^{Ac}	-19.3 ± 0.97^{ABb}	-18.4 ± 0.65^{Bc}	-19.7 ± 0.35^{ABc}

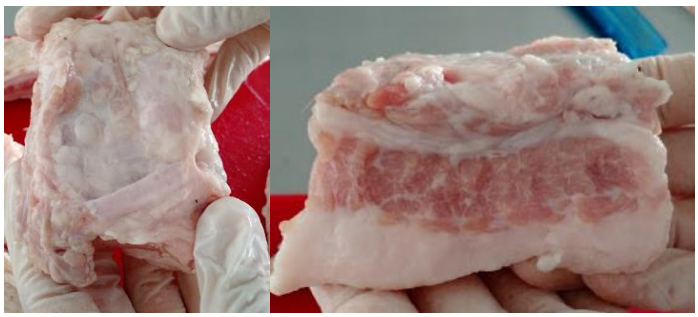
Appendix C. Images of the mucoadhesion test



a) Mucoadhesion test



B) Porcine buccal mucous with films before immersion in PBS, 37°C, 20rpm



C) Side of porcine buccal mucous and transversal cut of porcine cheek tissue