

Development of a new propolis microemulsion system for topical applications

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RESUMO: “Desenvolvimento de um novo sistema microemulsionado de própolis para aplicação tópica”. Sistemas microemulsionados (MES) oferecem inúmeras vantagens como liberadores de fármacos já que favorecem a absorção do princípio ativo e são, em muitos casos, mais eficientes do que outros métodos de liberação de substâncias bioativas. Neste trabalho, um MES foi obtido visando uma formulação para uso tópico, contendo extrato etanólico de própolis (PEE). Neste estudo, Diagramas de fase pseudoternários foram utilizados para caracterização das regiões de microemulsão, bem como para definição da região de Winsor IV do sistema PEE-MES (Tween 80 como tensoativo e álcool etílico como co-tensoativo, em baixo percentual). Os resultados obtidos indicaram que o melhor sistema microemulsionado contém C/S (cotensoativo/tensoativo) na razão 1,0, fornecendo uma microemulsão do tipo O/A com ampla região de Winsor IV. A formulação PEE-MES poderá vir a ser utilizada comercialmente para uso tópico no combate a inflamações de boca e garganta. Considerando o amplo uso de própolis em formulações convencionais, onde sua solubilidade representa um dos maiores problemas, o sistema PEE-MES disponibiliza este produto natural para aplicações farmacológicas, com boa solubilidade em um sistema microemulsionado do tipo O/A. Adicionalmente, o efeito bactericida do sistema PEE-MES foi comprovado e uma nova metodologia de extração de própolis é apresentada.

Unitermos: Própolis, sistema microemulsionado, formulação tópica.

ABSTRACT: Microemulsion systems (MES) offer advantages as drug delivery systems, among them favour drug absorption, being in most case more efficient than other methods in delivering of drug. In this work a new MES was obtained in order to be applied as a pressurized aerosol formulation containing bee propolis ethanolic extract (PEE). For that, pseudoternary phase diagrams were used to characterize the microemulsions boundaries and also to define the Winsor IV microemulsion region of the PEE-MES system containing Tween 80 as surfactant and the cosurfactant ethyl alcohol in small percentage. The obtained results indicated that the best MES was composed by Tween 80 and ethyl alcohol with C/S (cosurfactant/surfactant) ratio equal to 1.0, since it provided a large boundaries in the obtained O/W microemulsion region. This PEE-MES formulation, in which bee propolis consisting as oil phase, is herein designed for topical uses (PEE-MES spraying) in order to treat mouth and throat inflammatory infections. Considering the very large uses of bee propolis in conventional vehicles, MES type of delivery system has to be compatible with achieving the highest drug aim loadings, determined substantially by the specific MES application (drug solubilization in water systems) improving in this case, propolis pharmacological applications. Additionally, PEE-MES antibacterial effect was evidenced and the microemulsion system PEE-MES was also used as newest chemical approach for extraction of bee propolis material from resinous hive.

Keywords: Propolis, microemulsion system, topical formulation.

INTRODUCTION

Microemulsions are macroscopically single-phase, thermodynamically stable, multicomponent fluids, optical clarity, and isotropic mixtures of oil, water and surfactant, frequently in combination with a cosurfactant, consisting in uniform spherical droplets. Those systems nature either hydrophilic aggregates in oil [water-in-oil system (W/O)], hydrophobic aggregates in water [oil-in-water system (O/W)] and also bicontinuous (effectively continuous systems in both water and oil: W/O and O/W) in which the dispersed phase is in the nanometer size range, containing domains of nanometer dimensions stabilized by the interfacial film of surface active agents. Such systems usually form spontaneously, presenting structural entities much smaller than the wave length of light which is the reason for their transparency. In all these cases, the surfactant forms an interfacial film that separates the water and oil domains. The presence of the cosurfactant is often required in order to lower the interfacial tension of this interface because a low interfacial tension is essential for the formation of microemulsions systems (MES). Advantages associated with MES include spontaneous formation, enhanced solubilization capacity, easy obtaining and scale-up (Bagwe et al., 2001; Mehta & Bala, 2000; Rossi et al., 2007a).

In the past three decades microemulsions have been the focus of extensive research worldwide due to their importance in a variety of technological applications. These systems are currently of interest to pharmaceutical scientists because of their considerable potential to act as drug delivery vehicles by incorporating a wide range of drug molecules. Additionally, offer advantages as drug carrier systems improving drug bioavailability for oral, parenteral and pulmonary administration, and also for transdermal and ocular delivery (Chen et al., 2004; Cunha et al., 2003; Dantas et al., 2002; Lu et al., 2008; Lv et al., 2005; Lawrence & Rees, 2000, Oliveira et al., 2004; Rossi et al., 2006, 2007b; Valenta & Schultz, 2004).

It is noticeable from the literature that there has been a significant correlation among nature of surfactant (S) and cosurfactant (C) and also C/S ratio with large boundaries formations in O/W and W/O microemulsion regions. Microemulsion-based media using vegetable oils such as soybean, palm and ricin oil, were important for technological applications (Dantas et al., 2001; Lee et al., 1995).

Concerning to bee propolis its biological activities depends of its chemical composition which is correlated to factors such as season and vegetation region. Up to now, more than 300 compounds have been found in propolis. Among them were detected aminoacids, aldehydes, phenolic acid and esters, phenolic aldehydes, aliphatic acids, aromatic acids, aromatic acid esters, chalcones, dihydrochalcones, flavanones, flavones, flavonoids, hydrocarbons, fat esters, fat acids, terpenoids, steroids and

sugars (Bankova et al., 1992, 1995, 2000, 2002; Castro et al., 2007; Chang et al., 2008; Dutra et al., 2008; Greenaway et al., 1987, 1991; Lustosa et al., 2008; Marcucci, 1995; Nascimento et al., 2008; Pereira et al., 2002; Sousa et al., 2007). In addition, vitamins B1, B2, B6, C and E were found to be present. Inorganic elements such as copper, manganese, iron, calcium, aluminum, vanadium and silicium were also detected (Debuyser, 1983; Lustosa et al., 2008). New compounds have been isolated from Brazilian (3,5-diprenyl-4-hydroxycinnamic acid, among other phenolic compounds) and Chinese (octa-cosanol) samples of propolis (Castaldo & Capasso, 2002; Marcucci et al., 2001).

Bee propolis has been tested as counterpoison, tissue regenerator, anaesthetic, liver protectors, imunostimulants, antigerm, antifungus, antiprotozoan, antiviral antiinflammatory, antioxidant and anticancer agent (Dutra et al., 2008; Hegazi & Abd El Hady, 2002; Longhini et al., 2007; Lustosa et al., 2008; Simões et al., 2008). Due to its rich biological properties, propolis tinctures and extracts have become more and more used in the industry, founding utility in a wide range of nutritional, pharmaceutical and cosmetic applications, in products like creams, gels, hydroalcoholic solutions, soaps, pointing out some examples (Debuyser, 1983; Marcucci, 1995).

The purpose of the present study was to investigate the influence of bee propolis, a resinous hive product collected by honeybees (*Apis mellifera* specie) from various Brazilian plant sources, in microemulsion systems consisting of bee propolis ethanolic extract (PEE) as the oil phase. For that a new microemulsion system was obtained using Tween 80 as surfactant and ethyl alcohol as cosurfactant, in order to improve propolis pharmacological applications and also to design the obtained pharmaceutical formulation EEP-MES for topical uses to treat mouth and throat inflammatory infections.

MATERIAL AND METHODS

Chemicals

The used surfactants (Tween 20 and Tween 80) and cosurfactants (ethyl alcohol, isoamyl alcohol and propyleneglycol) were analytical grade (ACROS Organics).

Ethanolic extract of propolis

The propolis sample was obtained from regional producer in Rio Grande do Norte state of Brazil.

A 5% bee propolis solution was prepared as follows: 10 g of bee propolis was minced, all the visible particles of wax and other admixtures were removed. The remaining semi-purified material was submitted to extraction with ethyl alcohol (96%). The solution was infused during three days in a dark place, at room

temperature (stirred during 30 h). The surface layer of the obtained material got dark brown color with a pleasant odor. The medium layer consists of non dissolved particles of bee propolis and the lower layer consisting in a wax gray color material. In a filtered surface layer the amount of the dry substance was determined using a refractometer. For each 100 mL of bee propolis solution 5 g of dry substances were obtained.

Microemulsion region determination

To determine the microemulsion region in a pseudoternary phase diagram the oil phase (PEE) was mixed with specific cosurfactant (C) and surfactant (S) and the mixture was titrated with water until the mixture turned turbid (water volume recorded). Pseudoternary diagrams were constructed by plotting the amount of water, oil and cosurfactant/surfactant (C/S) components.

Microemulsion composition

The selected microemulsions regions to perform the experiments were composed by distilled water (Aqueous phase); bee propolis ethanolic extract (PEE oil phase-OP) and the specific C/S was defined by analyses of the surfactant and cosurfactant influences in the pseudoternary phase diagram.

Extraction process of propolis using MES

Microemulsions (O/W) compositions: a) PEE-MES-1: 23% of C/S (alcohol/Tween 80), 1% of FO [oil phase, which is PEE (propolis extracted by conventional extraction, as described above), 76% of FW (water phase); b) PEE-MES-2: 35% of C/S (alcohol/Tween 80), 0.5% of FO (PEE), 64% of FW; and c) PEE-MES-3: 50% of C/S (alcohol/Tween 80), 25% of FO (PEE: propolis extracted by conventional extraction, as described above), 25% of FW (water phase).

Ten grams of bee propolis was minced, all the visible particles of wax and other admixtures were removed. The remaining semi-purified material was submitted to extraction with those PEE-MES systems. The solution was infused at room temperature (stirred during 24 h). The surface layer of the obtained material got dark brown color with a pleasant odor. The medium layer consists of non dissolved particles of bee propolis and the lower layer consisting in a wax gray color material (the wax excess of PEE-MES was removed using ethanol) giving propolis in good percentages (69-78%).

Ethanolic extract of propolis (PEE) and PEE-MES chemical composition

Analytical HPLC (Merck-Hitachi, Germany) equipped with a pump (model L-6200, Merck-Hitachi,

Germany) and a diode array detector (L-6200, Merck-Hitachi, Germany). The elution was carried out with a linear gradient and a flow rate of 1 mL/min. The detection was monitored at 280 and 340 nm. The chemical identity of the extracted propolis was compared with previously reported works (Marcucci et al., 2001; Marcucci, 1995).

Studied activity

Antimicrobial activity was assayed using the microorganism *Staphylococcus aureus* (ATCC 13150). The assays were carried out using the technique described by Bauer et al. (1966). The cultures were incubated at 35 °C for 24 h, and then resuspended in saline solution in comparison to 1.0 of MacFarland scale standard. This suspension (100 µL) was placed on surface medium (Nutrient Agar). The discs of filter paper were individually saturated with samples and distributed on surface medium (5 discs/dish). The diameter of inhibition zone (mm) was measured after 24 h of incubation.

RESULTS AND DISCUSSION

Brazilian bee propolis has been the subject of an intensive study of chemists, biologists, and physicians due to its potential in folk medicine. Characteristically, it is a lipophilic material, that posses a pleasant aromatic smell, and vary in color, depending on its source and age, being hard and brittle when cold but soft, pliable, and very sticky when warm, hence the name bee glue (Marcucci, 1995).

In this study the two nonionic surfactants Tween 80 and Tween 20 were selected because of its hydrophilicity, in order to have larger oil in water (O/W) microemulsion region. The presence of the polyoxyethylene moiety makes these surfactants soluble or dispersible in water, favoring the formation of O/W microemulsions. Additionally, Tween-based microemulsions are largely used as drug delivery systems (Lawrence & Rees, 2000; Mehta & Bala, 2000). The present study was carried out with a cosurfactant/surfactant ratio (C/S) equal to 1.0. Figure 1 shows the pseudoternary phase diagrams of the microemulsion region for the systems composed by Tween 80 and Tween 20. As can be observed, Tween 80 provided a larger microemulsion region with the presence of small areas rich in both oil (PEE) and water, making possible to obtain microemulsified systems showing little percentages of C/S. Ethyl alcohol is a nonionic cosurfactant that was associated to the surfactant with the purpose of neutralizing the repulsive effect among the surfactant polar heads, allowing the formation of a membrane between the micelle and the continuous phase of the microemulsion. In this study other cosurfactants (isoamyl alcohol and propyleneglycol) were evaluated (C/S = 1.0 and 2.0). For propyleneglycol only the C/S = 2.0 was analyzed due to the foam formation when C/S = 1.0 was applied, making difficult to identify phase separation process. Only the system using ethyl

alcohol presented a larger O/W microemulsion region with the presence of small oil and water regions, making possible to obtain microemulsified system with little percentages of C/S. Figure 2 illustrates those obtained pseudoternary phase diagrams. It is important to point out that ethyl alcohol is well used in oral drug formulations but its content should be minimized (0.5 to 10%) in order to avoid collateral effects in the intraperitoneal treatments, as well as in oral ingestions (Ansel et al., 2000; Lawrence & Rees, 2000).

The C/S ratio is a very important factor in the microemulsion existence domain. The system used to promote the C/S ratio study was the one composed by Tween 80, as surfactant, and ethyl alcohol, as cosurfactant, in the following C/S ratios: 0.5, 1.0, 2.0, and 4.0. Figure 3 illustrates the obtained pseudoternary phase diagrams, in which the microemulsion region is decreased by increasing C/S ratio. This data was associated to the surfactant concentration decreasing, that reduced PEE solubilization in the aqueous phase. With C/S = 0.5 a good microemulsion region was observed but the system required a long stirring time to be solubilized and showed high viscosity, making its utility difficult. These results indicated that C/S=1.0 was the best microemulsion system, in which a larger microemulsion region was observed containing both oil and water small regions. It is important to obtain microemulsified systems (with little percentiles of C/S) containing higher amounts of water with good bee propolis solubilization (in percentages ranging from 1 to 6%). In that the lower amount of PEE was observed for the formulation PEE-MES-1 (23% of C/S, 1% PEE, 76% water) and the highest to PEE-MES-4 formulation, in which C/S was 36%, oil phase (PEE) was 5%, and water phase (distilled water) was 59%.

Nowadays, ethanol extract of bee propolis is the most common applied form but studies concerning to water extract of bee propolis are increasing. The use of alcohol

provides an unpleasant taste to the product and makes it inconvenient when applied in wounds (Nagai et al., 2003). Propolis extraction method using alcohol as solvent is widely reportedly, in which common chemical extraction process such as maceration and Soxhlet apparatus have been applied. In that, at the optimum operating conditions, the extraction efficiency can reach from 30 to 45%.

Extraction process using water-in-oil (W/O) microemulsion systems, commonly referred as reverse micelles, has been widely used in purification of biological macromolecules. According to Zhou et al. (2008), theoretically, this method can also be used to extract small molecules from natural sources. In fact, using cetyl trimethylammonium bromide (CTAB) reverse micelles it was possible to extract the biological natural product quercetin-3-O- β -galactosidepyranose a kind of flavonoid commonly called hyperoside, that has many biomedical functions, from the natural source *Hypericum perforatum* L. (Zhou et al., 2008).

In this work microemulsion systems (PEE-MES-1, 2 and 3) were applied for extraction of bee propolis from resinous hive. Table 1 shows the effectiveness of this method for propolis extractions, representing significant increasing for propolis obtaining. The observed extraction efficiency over than 60% [69% of extracted propolis for PEE-MES-1 (C/S 23%, FO 1%, FW 76%); 76% for PEE-MES-2 (C/S 35%, FO 0.5%, FW 64%); and 78% for PEE-MES-3 (C/S 50%, FO 25%, FW 25%)]. It seems clear that the higher effective formulation was bicontinuous microemulsion (W/O and O/W), in which 78% of propolis was extracted. PEE-MES-1 and PEE-MES-2 with excess of the aqueous phase characterizing an oil-in-water (O/W) system showed lower percentages of propolis extraction. The found results demonstrated that extraction using microemulsion is a very promising method to separate (and purify) materials of small molecules.

Effects of propolis on bacteria isolated from

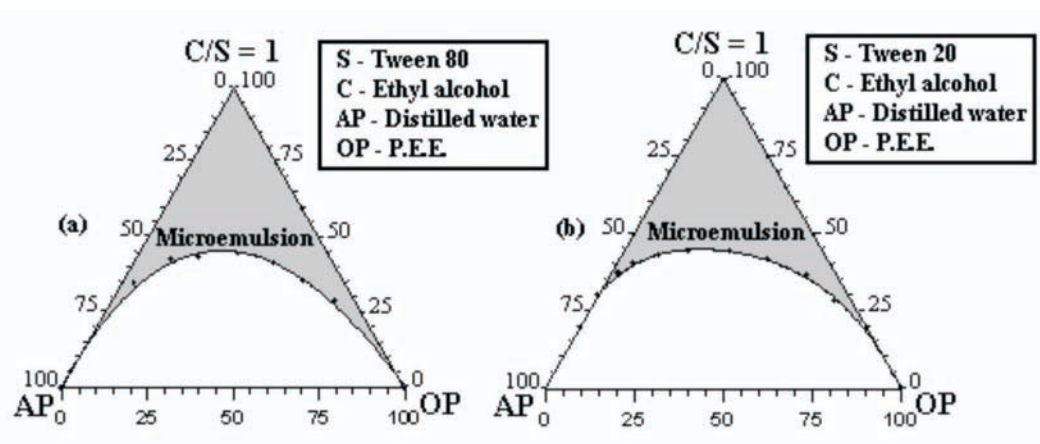


Figure 1. Pseudoternary phase diagrams showing microemulsion region for the systems composed by the surfactants (S) Tween 80 (a) and Tween 20 (b) ($T = 30\text{ }^{\circ}\text{C}$) with ethyl alcohol as cosurfactant (C) and bee propolis ethanolic extract (PEE) as the oil phase.

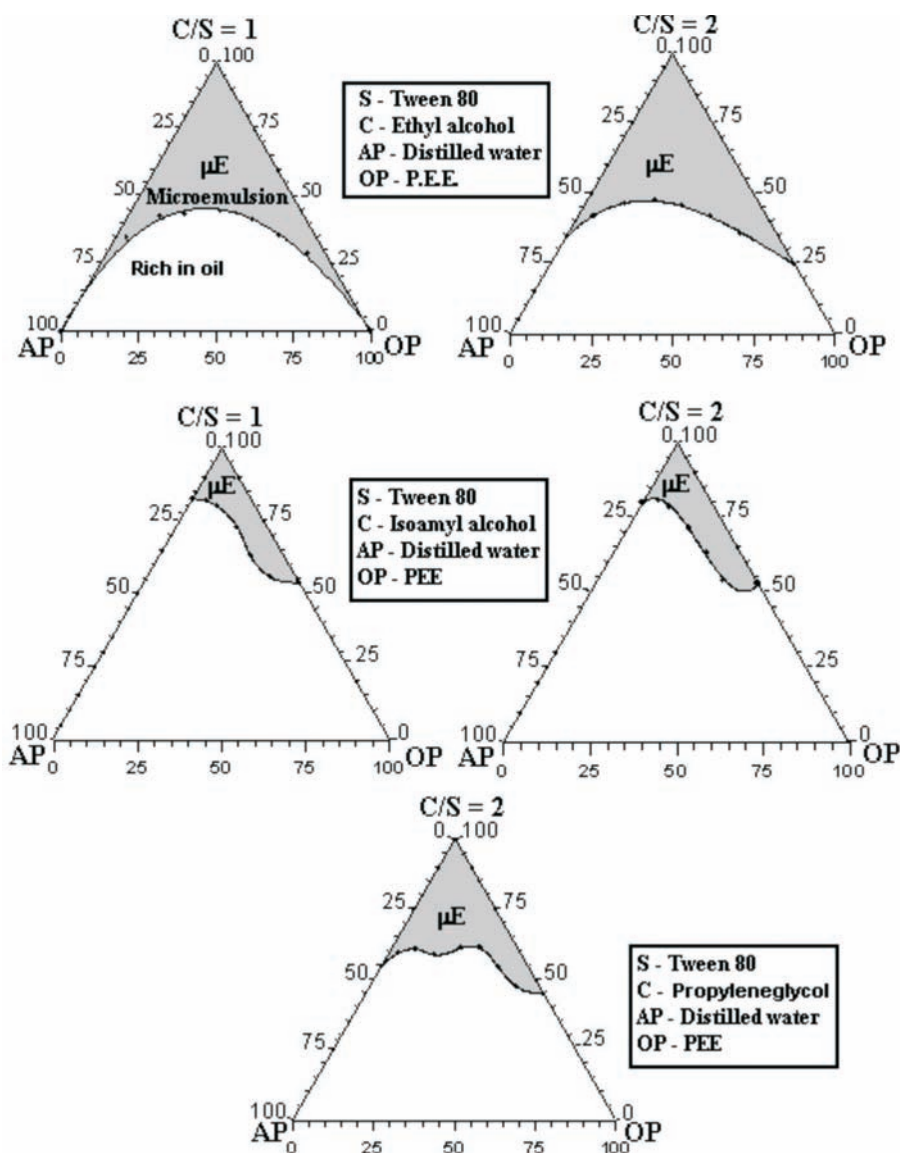


Figure 2. Pseudoternary phase diagrams showing the cosurfactants influences in microemulsion region (μE) for the systems composed by lipid microemulsions systems consisting of bee propolis ethanolic extract (PEE) as the oil phase and Tween 20/80 as surfactants ($T = 30\text{ }^{\circ}\text{C}$).

oral cavity showed that *Staphylococcus mutans* and *Lactobacillus* sp. afforded the most consistent results, followed by *Staphylococcus aureus* and *Staphylococcus epidermidis*. In this work the PEE-MES formulation [23% C/S (Tween 80/ethyl alcohol); 1% PEE; 76% aqueous phase] showed to be effective against the Gram-positive *Staphylococcus aureus* bacteria {MIC 71%, 10.3 ± 2 [zone of inhibition (mm)]; testes were done in triplicate; values are mean \pm S.D.}, which was susceptible to low propolis concentration (1% of PEE) present in this microemulsion formulation as oil phase. This result confirmed the moderate antibacterial activity of low concentrations of propolis extract against this specific microorganism (Marcucci et al., 2001; Sforzin et al., 2000). In other hand, rendered a consistent result confirming the biological action of PEE-MES. The bactericidal activity of PEE-MES was

correlated to the combined effect of several components that were herein identified in both PEE and PEE-MES by High Performance Liquid Chromatography (HPLC). The obtained HPLC analyses of the extract PEE and microemulsion formulation PEE-MES were quite similar, among the identified chemical components prenylated *p*-coumaric acids (phenolic type compounds) and their derivatives, additionally to other prenylated constituents (3-prenyl-4-hydroxycinnamic acid; 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran; 3,5-diprenyl-4-hydroxycinnamic acid; 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran) which were previously reported to be present in brazilian propolis samples (Marcucci et al., 2001; Bankova et al., 2000).

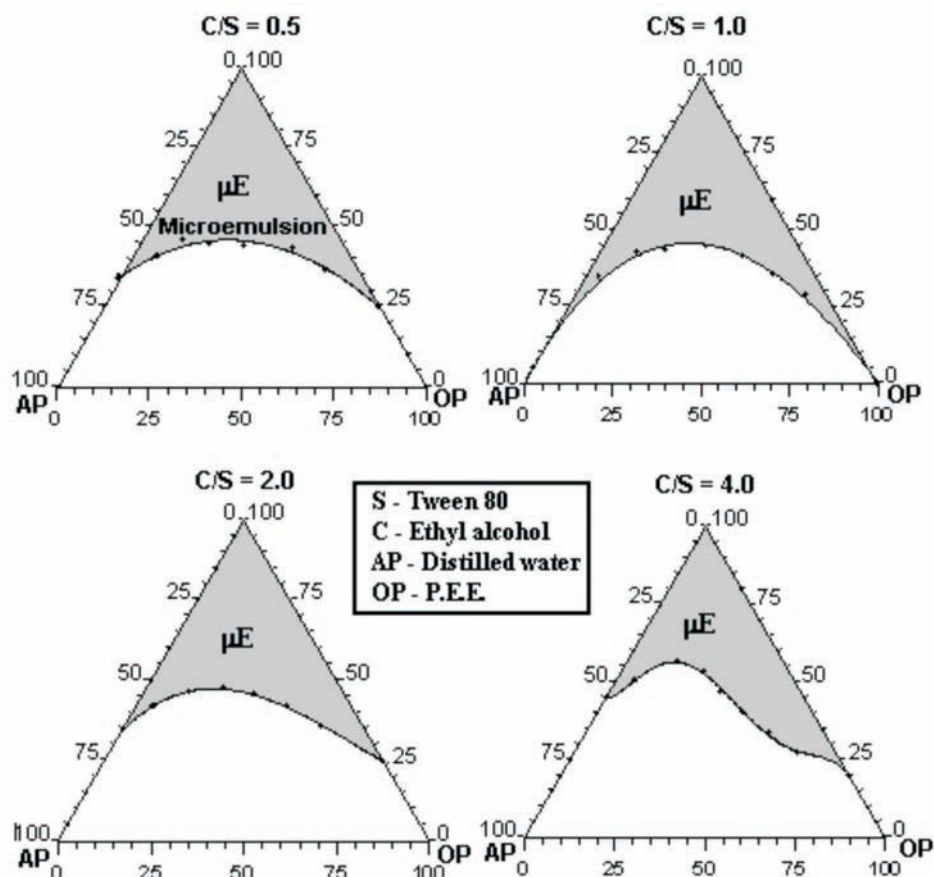


Figure 3. Pseudoternary phase diagrams showing microemulsion region (μE) for C/S ratios equal to 0.5, 1.0, 2.0, and 4.0 ($T = 30\text{ }^{\circ}\text{C}$).

Table 1. Composition of the microemulsion systems utilized in the PEE-MES extraction process.

MES-system	C/S (%)	FO (%)	FW (%)	Propolis Extraction (%)
PEE-MES-1	23	1	76	69
PEE-MES-2	35	0.5	64	76
PEE-MES-3	50	25	25	78

Cosurfactant (C)/Surfactant (S); C/S: (alcohol/Tween 80); FO (oil phase): propolis extracted obtained by conventional maceration method (PEE); FW (water phase)

CONCLUSIONS AND PERSPECTIVES

The development of this research allowed to conclude that the results of this study were very satisfactory, since microemulsified systems with propolis as oil phase can be produced with a water content ranging from 60 to 76% (oil-in-water system). Clearly, the use of O/W microemulsions in drug delivery is more used than W/O system. In fact, the droplet structure of O/W microemulsion is often retained on dilution by a biological aqueous phase, thereby permitting oral as well as parenteral administration. However, the process of dilution will result in the gradual desorption of surfactant located at the droplet interface. This process is thermodynamically

driven by the requirement of surfactant to maintain an aqueous phase concentration equivalent to its critical micelle concentration (CMC) under different conditions.

In this work the formulation PEE-MES provided a starting point from which suitable biological oils might be selected for formulation into pharmaceutically acceptable microemulsions. Representing a newest natural source of exploration for microemulsion based media, since vegetable oils are not common to be cited in the literature as oil phase of microemulsions pharmaceutical formulations.

The new formulation PEE-MES represents an important oral drug delivery offering microemulsions advantages such as: favor drug absorption, in most cases faster and more efficient than other nanotechnological formulations in delivering the same amount of drug. From PEE-MES a novel pressurized aerosol system could be devised for topical uses in order to treat mouth and throat inflammatory infections caused by oral cavity bacteria (as an example). In that, PEE-MES showed to be effective against the Gram-positive *Staphylococcus aureus* bacteria.

The formulation type PEE-MES in which propolis is the oil phase was utilized as a new propolis process extraction representing oil-in-water (O/W) systems (PEE-MES-1 and PEE-MES-2) as well as a bicontinuous (W/O

and O/W) system (PEE-MES-3). At the optimum operating conditions, the extraction efficiency can reach 69 to 78% of propolis crude material, been significantly more effective than the conventional methods for propolis extraction. This result was coherent with the work developed by Zhou et al. (2008) in which a bioactive flavonol was extracted (in great amount) using reverse micelles (W/O system).

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